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Emerging Approaches for Ocular Surface Regeneration

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Abstract

Purpose of review: In this manuscript, the recent advancements and novel approaches for regeneration of the ocular surface are summarized.

Recent findings: Following severe injuries, persistent inflammation can alter the rehabilitative capability of the ocular surface environment. Limbal stem cell deficiency (LSCD) is one of the most characterized ocular surface disorders mediated by deficiency and/or dysfunction of the limbal epithelial stem cells (LESCs) located in the limbal niche. Currently, the most advanced approach for revitalizing the ocular surface and limbal niche is based on transplantation of limbal tissues harboring LESCs. Emerging approaches have focused on restoring the ocular surface microenvironment using (1) cell-based therapies including cells with capabilities to support the LESCs and modulate the inflammation, e.g., mesenchymal stem cells (MSCs), (2) bio-active extracellular matrices from decellularized tissues, and/or purified/synthetic molecules to regenerate the microenvironment structure, and (3) soluble cytokine/growth factor cocktails to revive the signaling pathways.

Summary: Ocular surface/limbal environment revitalization provide promising approaches for regeneration of the ocular surface.

Keywords

Ocular Surface Regeneration; Corneal Epithelium; Limbal Stem Cell Niche; Limbal Epithelial Stem Cell Deficiency; Extracellular Matrix; Mesenchymal Stem Cells

1. Introduction: Ocular Surface Homeostasis, Pathologies, and Regeneration

The ocular surface is the outermost layer of the eye including the tear film. It is constructed and protected by structural and functional modules which have highly-regulated cross-talks. These components include the tear film, cornea, conjunctiva, lacrimal glands, meibomian glands, eyelids and nerves [1]. Many of the interactions on the ocular surface are modulated

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by the immune system. Many ocular surface disorders occur following alteration of the balance in immune system regulation [2]. The resulting persistent inflammation due to immune dysregulation limits the regenerative potential of the ocular surface micro-environment. Thus, local stem cells lose their capacity for proliferation, migration and differentiation, which are vital for rehabilitation of the ocular surface.

The limbal niche is the microenvironment supporting the function of LESCs [3]. It consists of a specialized extracellular matrix, signaling molecules and cells including immune cells, mesenchymal cells, melanocytes, nerve and vascular cells [4]. Injuries to the limbal niche or LESCs may lead to a condition known as limbal stem cell deficiency (LSCD) where the corneal epithelium can no longer be regenerated properly. This can have many consequences including non-healing epithelial defects, corneal neovascularization and opacification [5, 6]. Significant limbal injuries are almost always accompanied by the migration of immune cells in the area and subsequent inflammation [3]. The characteristics of an injured limbal niche include up-regulation of inflammatory and angiogenic factors such as IL-1α, IL-1β, IL-1 RA, IL-6, VEGF, ICAM-1, and VCAM-1. The persistent inflammatory environment in LSCD leads to additional recruitment of immune cells to the area and secretion of more inflammatory cytokines as well as impaired function of immune cells. The recruited immune cells secrete soluble pro-inflammatory factors, and the macrophages lose their ability to phagocytose [6, 7]. Additionally, macrophages stimulate adaptive T-lymphocytes, which worsens pathological inflammation [8]. Persistent inflammation in turn alters the expression of LESC stem cell markers, remodels the extracellular matrix, and disturbs the density and morphology of supporting cells in the limbal niche [5, 6]. Based on these observations, it can be concluded that the ocular surface microenvironment is disturbed in the setting of severe diseases, which in turn impairs regenerative mechanisms [9–13]. Therefore, in addition to repopulation of stem cells, emerging strategies are focusing on restoration of the limbal stem cell niche.

In this review, novel approaches for regeneration of the ocular surface based on repopulation of LESCs and restoration of the ocular surface/limbal microenvironment are summarized (Figure 1).

2. Cell-based Therapeutic Approaches

2.1. Epithelial Cells

Replacing deficient corneal epithelial cells is currently the most advanced approach available for regenerating the ocular surface. Several protocols have been studied including limbal tissue transplantation, cultivated limbal epithelial transplantation (CLET) and non-limbal epithelial cell transplantation. Limbal tissue transplantation is a surgical method that does not require cell-culture laboratory facilities. Current limbal tissue transplantation techniques consist of conjunctival limbal autograft (CLAU) [14], kerato-limbal allograft (KLAL) [15, 16], conjunctival limbal allograft (CLAL) [15], and more recently, simple limbal epithelial transplantation (SLET) [9, 10]. The limbal tissue can be harvested from donors including the fellow-healthy eye, cadavers, and living individuals. The dissected limbal tissue is placed on the damaged limbal area and is fixed by sutures or glue. The transplanted LESCs help restore corneal epithelial cell phenotype [17].

In cultivated limbal epithelial transplantation (CLET), the LESCs are collected from donors and expanded in the laboratory with or without feeder layers such as 3T3-fibroblasts and with or without a substrate carrier such as amniotic membrane. The resulting epithelial sheet is then transplanted to the diseased eye [11, 18]. Recent novel feeder layers for cultivation of LESCs include mesenchymal stem cells (MSCs), and limbal melanocytes [19, 20]. Xeno-free and serum-free protocols have also developed to decrease the chance of animal infectious transmission [21].

On the other hand, newer approaches have focused on the administration of epithelial cells derived from non-limbal sources. Cultivated oral mucosal epithelial transplantation (COMET) is one of the proposed treatments for reconstruction of the corneal surface in LSCD patients [22–24]. However, the phenotype of epithelial cells after COMET remains oral mucosal which results in thicker and opaquer epithelium with sub-optimal visual outcomes [24, 25]. Conjunctival epithelial cells [22, 23], amniotic epithelial cells [26] and differentiated epithelial cells from stem cells [27] are other potential sources which are under investigation for regeneration of ocular surface. For instance, Oyuyang et al. showed that limbal stem-cell like cells could be generated by transduction of PAX6 transcription factor in skin epithelial stem cells. Transplantation of these differentiated limbal stem-cell like cells to rabbit model of corneal epithelial stem cells deficiency led to regeneration of ocular surface [28]. Moreover, Hayashi et al. generated self-formed ectodermal autonomous multi-zones (SEAMs) of ocular cells from human induced pluripotent stem (iPS) cells using a differentiation culture medium and then induced differentiation of corneal epithelial cells by treating SEAMs with corneal epithelial differentiation medium. The produced human iPS cell-derived corneal epithelial cells repaired corneal epithelial defects in an animal model following transplantation [29].

Successful clinical translation of these approaches might improve the issues with allogeneic limbal tissue shortage and systemic immunosuppression; however, the lack of long-term clinical results and lack of corneal epithelial phenotype restoration in COMET, as well as cost, safety, and technical difficulties for administration of epithelial cells differentiated from iPS cells, may limit their promising wide application.

2.2. Mesenchymal Stromal/Stem Cells

Mesenchymal stromal/stem cells (MSCs) are multipotent stem cells present in almost all tissues of the body, in part providing support to regional stem cells that are responsible for replacing damaged cells. MSCs also have immune-modulatory effects by secreting anti-inflammatory cytokines/growth factors, and thus have been studied for cell-therapy for their immune-modulating and regenerative properties. MSCs derived from bone marrow (BM-MSCs) are the most characterized and administrated stem cells for clinical applications. The immunomodulatory effects of the MSCs have shown to involve various cell-surface molecules such as CXCR4, CD44, integrins, ICAM, CD90, and CD105 or secreted factors including TGF-β, IL-10, Leukemia inhibitory factor, semaphoring-3A and TSG-6 [30]. Several studies have shown the healing effects of MSCs in ocular surface disease models, e.g., chemical-burns [31], dry eye syndrome [32], and corneal transplantation [33]. For instance, periorbital injection of bone marrow-MSCs in a murine model of dry eye syndrome

resulted in reduction in infiltration, proliferation and differentiation of CD4+ T cells, attenuation of Il-2 and IFN-γ-inflammatory factors-, increase in aqueous tear production and conjunctival goblet cells [32]. Furthermore, administration of bone marrow-MSCs incorporated in a polysaccharide hydrogel on the ocular surface in a rat alkali-burn model led to healing of the corneal epithelium with less opacity and neovascularization than controls. Also, anti-inflammatory and anti-angiogenic cytokines were up-regulated, whereas, chemotactic and angiogenic factors were attenuated [34]. Therefore, the primary underlying mechanism of BM-MSCs healing effects in corneal damages is modulating the function of immune cells in turn promoting a more regenerative environment.

Recent studies have shown the presence of MSCs in the limbus. Observations made by in vivo confocal microscopy (IVCM) revealed that L-MSCs (a.k.a, limbal/corneal stromal stem cells) are located in clusters at anterior limbal stroma adjacent to LESCs [35] where they make direct physical contact with LESCs in the limbal niche. The interaction of L-MSCs and LESCs are mediated by various signaling molecules e.g. aquaporin-1 and vimentin [36], chondroitin sulfate (6C3 motif) [37], SDF-1/CXCR4 [38], BMP/Wnt [39], and IL-6/STAT3 [40]. Paracrine factors also facilitate the interactions between L-MSCs and LESCs [19]. L-MSCs have been extracted using digestion or explant based methods and found to fulfill the minimum criteria of human MSC characterization [41], defined by International Society of Cell Therapy, including plastic adherence, differentiation to adipocytes, chondrocytes and osteocytes and also the expression of CD105, CD73 and CD90 in addition to lack of CD14, CD34, CD 45 and HLA-DR expression [42]. L-MSCs have similar characteristics to BM-MSCs in terms of immunomodulatory and anti-inflammatory effects [41, 42]. Although the majority of healing effects by MSCs obtained from various sources are mediated by modulating innate and adaptive immune system, there are minor functional differences related to MSCs' tissue-of-origin [43, 44]. For instance, our team showed that L-MSCs produce soluble fms-like tyrosine kinase-1 (sFLT-1), a well-known antiangiogenic factor. BM-MSCs do not produce sFLT-1, however they still demonstrate anti-angiogenic effects when applied to the cornea due given their anti-inflammatory properties [41].

Administration of L-MSCs in corneal injury models led to improvement in corneal epithelial wound healing. For instance, sub-conjunctivital injection of human L-MSCs in rabbit models of alkali-burn improved corneal epithelial wound healing and attenuated corneal neovascularization, number of goblet cells, and corneal opacity [45]. A pilot clinical study showed that administration of L-MSCs using fibrin sealant in five patients with acute corneal burns, non-healing ulcers and post-keratitis scars, improved visual acuity, enhanced corneal clarity and reduced corneal vascularization up to one year follow-up in comparison with matched controls [46, 47]. Administration of MSCs incorporated in fabricated extracellular matrix is a promising approach for regenerating the ocular surface in moderate to severe disease.

Given the promising results of local MSC administration for ocular surface regeneration in pre-clinical large animal models, clinical applications are being pursued for patients with severe surface disease. Bone marrow-MSCs have the advantage of being more advanced in clinical development as they have been infused intravenously in numerous patients for non-

ocular indications. Thus, their clinical administration for ocular surface regeneration may be considered as a natural extension of these studies.

2.3. Melanocytes

Melanocytes are responsible for pigmentation of the PVs and protect the limbal stem cells against UV radiations by producing melanin. Recent studies have shown that limbal melanocytes may have other supportive functions in the limbus [48–50]. Limbal melanocytes locate in the compact clusters of limbal epithelial cells in limbus and have direct contact with limbal epithelial stem cells mediated by N-, P- and E-cadherins and L1-CAM. These cells have been extracted successfully from human cadaveric corneal tissues. Limbal melanocytes provide efficient support of limbal epithelial stem cells in vitro. The epithelial cells co-cultured with human limbal melanocytes retain their stemness proved by high expression of stem cell markers, e.g., CK15, Bmi-1, and p63 and deficient expression of CK3 differentiation marker. Limbal melanocytes have shown comparable effects regarding supporting the limbal epithelial cells as 3T3 fibroblasts and limbal mesenchymal stem cells. They also inhibit activated T-cells and vascular endothelial cells which is useful in regenerating the ocular surface and limbal niche. Moreover, a three-dimensional culture of human melanocytes with limbal epithelial cells enhanced the development of multi-layered epithelial sheets, whereas the basal layer remained undifferentiated [48–50]. The potential therapeutic effects of limbal melanocytes have just emerged into the field and further in vitro and in vivo studies are still needed to elucidate their regenerative capacity.

3. Extracellular Matrix-based Approaches

The extracellular matrix (ECM) is fundamental for physiological renewal of the ocular surface/limbal microenvironment. The limbal ECM is composed of structural proteins and adhesive elements [49]. The physical organization of limbal niche crypts and its basement membrane consists of various collagens, laminins, fibronectin, and chondroitin sulfate [51]. It has been reported that the limbal niche stromal ECM regulates epithelial differentiation, proliferation, and apoptosis of LESCs [52]. Therefore, regenerative approaches to revitalize the extracellular matrix have the potential of restoring the proper function of the limbal niche.

3.1. Amniotic Membrane

Amniotic membrane (AM) is the conventional ECM used for the management of ocular surface disorders. AM is obtained by peeling off the fetal membranes. It has no innervation and vascularization and has appropriate transparency [53]. The healing effects of AM are attributed to its rich extracellular matrix. The AM-ECM contains collagen, fibronectin, and laminin as well as growth factors such as epidermal growth factor (EGF) and hepatocyte growth factor [54]. Besides the innate healing capability of the AM for regeneration of the ocular surface, it is a proper carrier for delivery of different cells on the ocular surface. The collagen-rich structure of the AM provides the potential of fabricating scaffolds for tissue engineering purposes [55].

3.2. Bio-active Hydrogels

An attractive emerging approach to regenerate the ocular surface is fabrication of bio-active hydrogels which could be applied with/without cells. These hydrogels mimic the proper ECM for cell proliferation, migration, and differentiation. Protocols for production of these hydrogels are mainly based on decellularization and digestion of animal tissues or application of commercially available purified structural proteins [56]. Porcine corneas have been decellularized and digested to produce a bio-active hydrogel for regeneration of ocular surface after injuries. This hydrogel is compatible with epithelial and stromal cells; thus, it is a proper candidate for ocular surface cell delivery approaches [57, 58]. The mixture of collagen, hyaluronic acid, laminin, and elastin also provide an appropriate hydrogel for ocular surface regeneration [59]. These combinations are also proper sources for application in three-dimensional bioprinting of structures applicable in ocular surface regeneration [60, 61]. Other biological materials have also been used for production of bio-active ECMs supporting the stability of the ocular surface, such as fibrin [62], silk fibroin [63], and biocompatible polymers [64]. For instance, it has been shown that silk-derived protein enhances migration, adhesion and proliferation of corneal epithelial cells [65]. Moreover, the silk film could be patterned to navigate the corneal epithelial cells behavior by changing their gene expressions, so it could be customized for ocular surface repair [66]. A novel approach is to apply modified collagen hydrogels on diseased ocular surface which can be cross-linked in situ. The idea of this approach is to apply hydrogels containing keratocytes to replenish the damaged corneal stroma to provide proper support for epithelial growth [67].

3.3. Fabricated Extracellular Matrices

Another strategy for regeneration of ocular surface/limbal microenvironment ECM is to fabricate a proper ECM for epithelial (stem) cell homing, proliferation, migration and differentiation. Many approaches have been proposed for the production of the desired ECM. Efficient decellularization of human and/or animal corneas with preserving the integrity and functionality of the native ECM have been taken into consideration recent years. Several protocols have proposed decellularization of human and animal corneas including freeze-thawing, freeze-drying, detergent treatment, and acid/base treatment [68, 69]. Our group previously showed that treatment of human cadaveric corneal tissues with hypertonic Sodium Chloride solution followed by nuclease treatment results in efficient cell content removal while preserving an intact ECM. The decellularized human corneas supported the growth of corneal epithelial and fibroblast cells [70]. Furthermore, transplantation of decellularized human limbal ECM promoted epithelialization and inhibited haze formation in a rat partial limbal injury model [71].

4. Therapeutic Factors

Cellular components and signaling pathways are essential modalities in preserving the homeostasis of the ocular surface. Revitalizing the function of disrupted ocular surface micro-environment by local delivery of exogenous growth factors provides another novel approach for ocular surface regeneration [3, 72]. There are a number of potential sources for obtaining therapeutic factors for the ocular surface: hemoderivatives [73–75], soluble recombinant growth factors [76], derivatives from amniotic membrane [77], and secretions

from cells with healing characteristics [41, 42, 78]. The emerging therapeutic factors for ocular surface regeneration are discussed in the following sections.

4.1. Hemoderivatives

Blood-derived eye drops have become increasingly popular in the setting of ocular surface diseases. Platelet-derived preparations and autologous/allogeneic serum eye-drops (ASE) are used therapeutically for restoring the disturbed micro-environment of the ocular surface. They are rich in growth factors, cytokines, vitamins and minerals which are required for normal corneal epithelial homeostasis and can stimulate proliferation, differentiation, and migration on the ocular surface [79]. Autologous serum eye-drops are similar to human tears regarding components such as EGF, TGF-β, fibronectin, vitamin A and other common cytokines [73–75]. The clinical applications of ASE to the ocular surface range from dry eye and Sjögren disease to severe conditions including, graft-versus-host disease and keratoconjunctivitis [73, 80, 81]. [82].

Although successful rehabilitation of corneal pathologies after administration of ASE has been reported [80, 81], they have their limiting drawbacks. Imperfect stability, increased risk of infections during prolonged use, no standardized manufacturing and application protocols are some of the issues with the use of these products [83].

Platelet releasate (PR), plasma rich in growth factors (PRGF), and platelet-rich plasma (PRP) are produced from the supernatant of anti-coagulated whole blood. They are rich in some growth factors (e.g., EGF, TGF, PDEF, bFGF, and IGF-1) and are prepared by different protocols. Although different outcomes regarding various preparation protocols have been reported, in vivo and clinical experiments have shown the potential regenerative and reconstructive capability of platelet-derived products to be comparable to ASE [84, 85]. Moreover, a recent randomized clinical trial evaluated the effects of PRP injection in patients with severe dry eye disease. The results indicate improvements in corneal staining, mean Schirmer value and tear break-up time after PRP injection after 90 days of follow-up [86].

4.2. Soluble Growth Factors

Soluble growth factors with neuroprotective, anti-inflammatory and antiangiogenic properties have been used for ocular surface regeneration. These can be extracted from cell secretomes [87, 41, 42], and/or produced by recombinant techniques [88, 89].

Pigment epithelial-derived factor (PEDF) is extracted from human plasma and can be used in the ocular surface regeneration. The effect of PEDF and PEDF-derived factors in supporting stem cell survival and maintaining their multi-potency has been described [76]. Moreover, ciliary neurotrophic factor and IL-1 receptor antagonist peptide could also be considered as potential regenerative factors in ocular surface diseases [88, 89].

Nerve growth factor (NGF) is a soluble growth factor, which plays a crucial role in the developing and maintaining the visual system as well as controlling pathologic conditions [90]. Under physiological conditions, NGF is secreted in the aqueous humor and expressed in the anterior segment [91]. The positive effects of topical NGF in modulating the corneal and conjunctival healing, in animal models and patients with severe ocular surface diseases

such as neurotrophic and autoimmune corneal ulcers, have been reported [92]. The potential application of topical NGF in dry eye, rejected corneal transplantation and herpetic keratitis is also demonstrated [93–95]. In a phase II double blinded, multicenter randomized trial, 156 patients with stage 2 or stage 3 NK in one eye were recuritied and randomized in 3 equal-number groups treated with recombinant human NGF (rhNGF) 10 μg/ml, 20 μg/ml, or vehicle. The 4- and 8-weeks follow-up results showed that rhNGF had led to significant corneal wound healing compared to vehicle. Moreover, 96% of patients remained corneal wound-free after 48- and/or 56-weeks follow-ups with tolerable adverse effects [96]. rhNGF was recently approved by FDA for ocular surface treatment in patients with moderate to severe NK. This achievement in clinical translation of a therapeutic growth factor, sets the stage for other ocular surface regenerative therapies.

4.3. Amniotic Membrane Derivatives

Derivatives from Human Amniotic Membrane (HAM) have been used in ocular surface restoration [97]. Amniotic membrane extract eye-drop (AMEED) is a cocktail of HAM factors which has beneficial effects for in vivo cultivation of limbal stem cells in LSCD patients [77]. Clinical trials assessing the safety and efficacy of AMEED are currently underway [98].

Likewise, HC-HA/PTX3 is a potential factor useful for revitalization of ocular surface homeostasis. It is derived from HAM and contains heavy chain 1 (HC1) of inter-α-trypsin inhibitor which bonds to hyaluronan (HA) [99]. The beneficial effect of HC-HA/PTX3 complex for maintaining the LESCs self-renewal via modulating Wnt/BMP signaling in 3D-culture system has been demonstrated [100].

4.4. Cell Secretions (Secretomes/Conditioned Media, Exosomes)

Secretomes/conditioned media is the cocktail of secreted factors from in vitro cultivated cells. Mesenchymal stem cell conditioned media contains a combination of growth factors that can help regenerate the ocular surface [41, 42]. Both bone-marrow and limbal derived MSCs-secretomes have healing effects on corneal epithelial cells and modulate the immune system. The conditioned media of human bone-marrow MSCs contain high amount of the IL-1 Receptor Antagonist (IL-1RA) and also significantly reduce the expression of IL-1α and IL-1β in human corneal epithelial cells [101]. Moreover, lyophilized secretome of human bone marrow MSCs have synergistic effects with hyaluronic acid and chondroitin sulfate in facilitating corneal wound healing following mechanical epithelial injury and also ocular surface regeneration following alkali-burn in animal models. It is suggested that human BM-MSCs secretome upregulate the expression of CD44 receptors and enhance the binding of hyaluronic acid to CD44 [78].

Similarly, limbal mesenchymal stem cell conditioned media was shown to accelerate epithelial wound healing and decrease angiogenesis via soluble fms-like tyrosine kinase-1 (sFLT-1) and PEDF [41]. It diminishes and modulates the pathologic role of macrophages in developing corneal neovascularization and immunophenotype function, respectively [42].

Exosomes are nano-vesicles containing bioactive molecules such as microRNAs, mRNAs, and transcription and growth factors responsible for cell-cell communications [102, 103].

The size of exosomes is 40 to 100 nm, and while they fuse to the target cell membrane, their internal contents would be released and lead to cell phenotype change. The critical role of corneal epithelial cell exosomes in wound healing and angiogenesis have been already reported [103]. The potential therapeutic effects of L-MSCs-derived exosomes in both in vitro and in vivo models have been demonstrated by our team [104]. We showed that nanovesicles extracted from cultivated L-MSCs express CD9, CD63, and CD81 as the characterizing markers of exosomes. The isolated exosomes were uptaken by corneal epithelial cells both in vitro and in vivo and released their contents inside the cells. In vitro wound healing assays reveled that L-MSCs exosomes promote migration and proliferation of cultivated human corneal epithelial cells. Moreover, the corneal epithelial wounds in rodent models healed faster by treating with L-MSCs exosomes [104].

5. Summary and Conclusions

Major injuries or insults to the cornea can lead to significant loss of function on the ocular surface, which is primarily due to the disruption of its “regenerative” environment. Emerging strategies for regeneration of the ocular surface have focused on restoring a healthy and “regenerative” environment by replacing the deficient cells (e.g. limbal stem cells), applying immunomodulatory and regenerative cells and/or secretomes (e.g. MSCs) and reconstructing the ECM (e.g. hydrogels).

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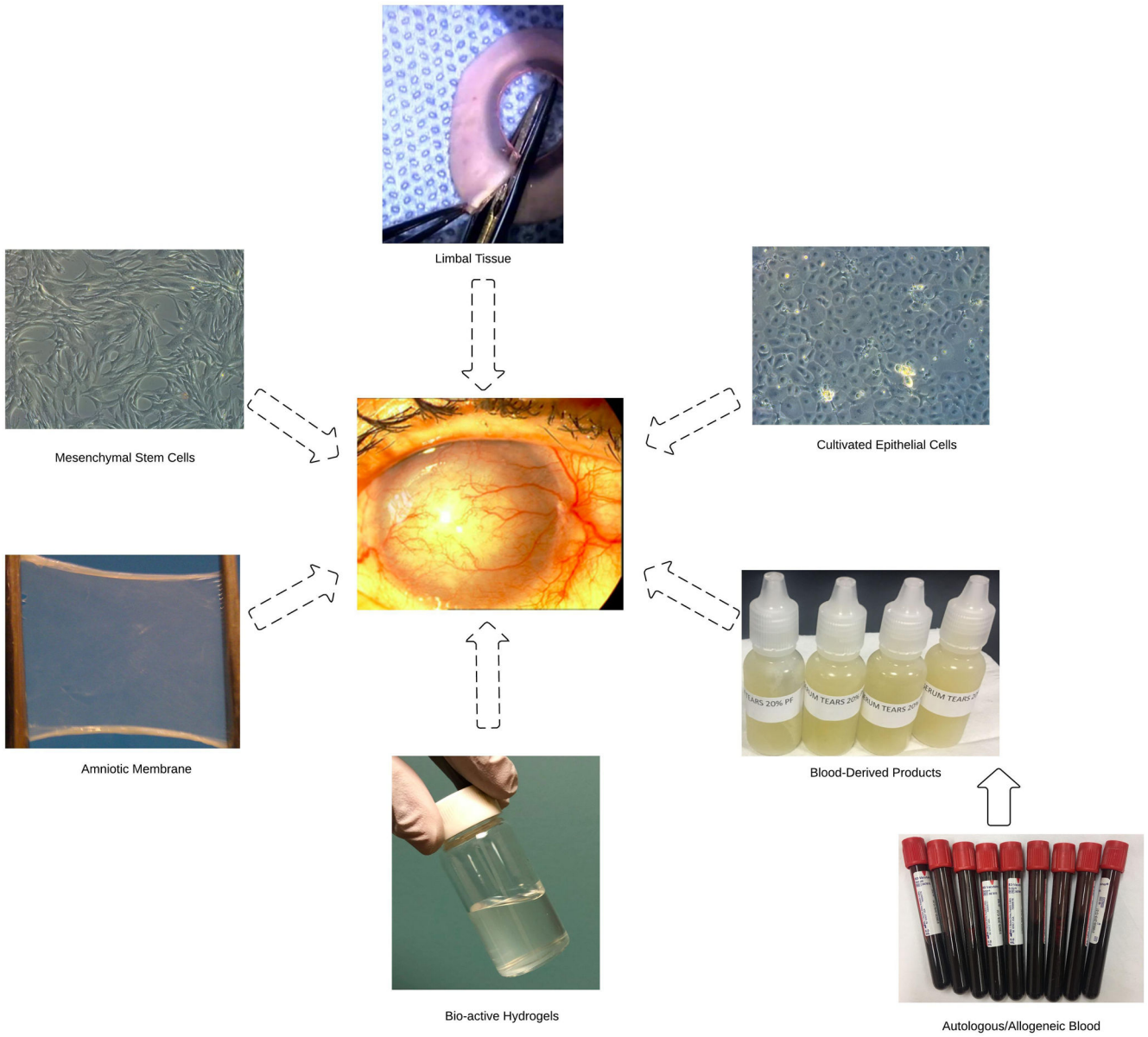

Figure 1:
Summary of emerging approaches for regeneration of the ocular surface.