UNIVERSITÀ DEGLI STUDI DI UDINE

CORSO DI DOTTORATO DI RICERCA IN
SCIENZE E TECNOLOGIE CLINICHE
CICLO XXIV

TESI DI DOTTORATO DI RICERCA

HUMAN ADIPOSE DERIVED STEM CELLS FOR CORNEAL DISORDERS

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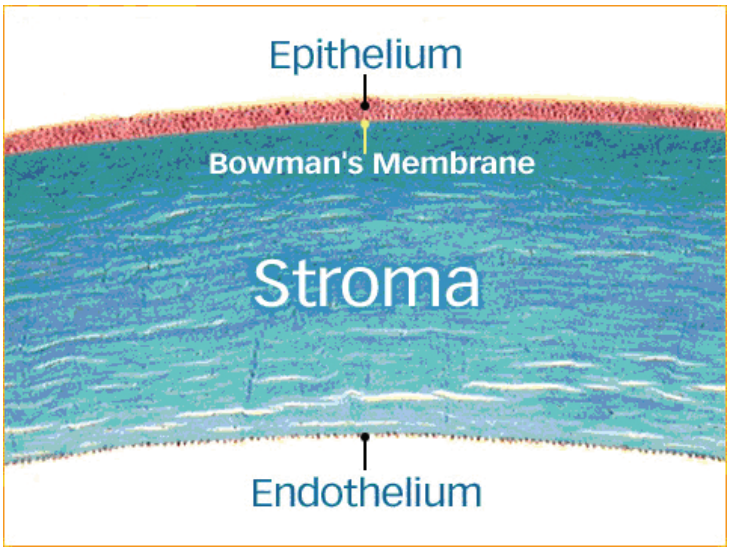
1. ANATOMY OF THE CORNEA

One-sixth of the outer layer of the eye forms the cornea, which is a transparent spherically shaped structure that serves as the outer window of the eye and covers the iris, pupil and anterior chamber. The cornea is the most important structure of the ocular surface that allows light entering the eye to focus onto the retina for good visual acuity, together with the crystalline lens. It accounts for almost 2/3 of the refractive optical power.

The cornea is mostly made up of connective tissue, composed of a thin layer of epithelium on the surface, a monolayer of endothelium facing the posterior inside part of the eye and a mid portion stroma in between. The transparency of the cornea is mainly because there are no blood vessels and very little cells within the stroma. Moreover, the morphology of the collagen fibers within the stroma are arranged in such away to preserve transparency. Infection, lesions, inflammation and other nocive factors can disrupt this delicate homeostasis within the cornea and even cause neovascularization, which can lead to opacification and resulting vision loss. Although the cornea is free from blood vessels, it contains the highest concentration of nerve fibers of any body structure, making it extremely sensitive to pain. The nerve fibers enter from the margins of the cornea and radiate toward the center. These fibers are associated with numerous pain receptors that have a very low threshold.

The cornea is composed of 5 layers, which include (from anterior to posterior):

1. Epithelium
2, Bowman’s membrane
3. Stroma
4. Descemet’s membrane
5. Endothelium

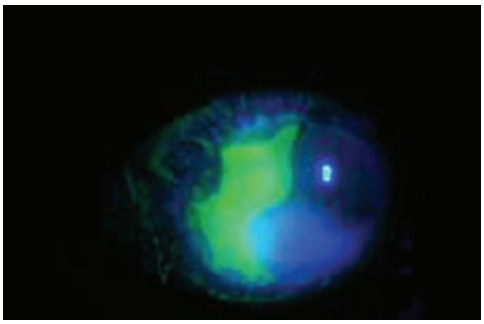


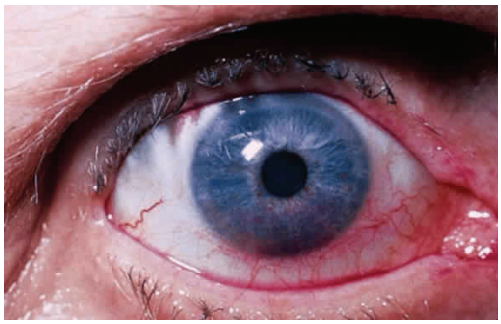
- **Corneal epithelium:**

The embryological derivation of the corneal epithelium is from the surface ectoderm at about 6 weeks of gestation. A healthy epithelium is needed to maintain a proper refractive surface, and protect the eye against infection and structural damage. It is composed of a thin epithelial multicellular tissue layer made of non-keratinized, non-secretory, stratified squamous epithelium cells, about 4-6 layers thick and about 40-50 μm in thickness. These cells are fast-growing and easily regenerated and need to be kept constantly lubricated; a function served by tears. The tear film is important in maintaining a healthy anterior surface. Irritation and edema of the corneal epithelium disrupts the smoothness of the air-tear film interface, which is the most significant component of the refractive power of the eye, thereby reducing visual acuity. The corneal epithelium cells are continuous with the conjunctival epithelium, and are shed constantly on the exposed layer and regenerated by multiplication in the basal layer.

Corneal epithelium cells undergo complete turn over that includes involution, apoptosis and desquamation every 7-10 days. The most superficial layers of epithelium form 2-3 flat layers formed of polygonal cells, which have extensive microvilli and

microplicae on the anterior surface. The cells are joined by tight junction complexes that restrict tears from going past the intercellular junctions. Routine ocular surface assessment in a clinical setting involves tests with dyes like fluorescein and rose Bengal, which are repelled and do not remain on a healthy and intact epithelium surface.



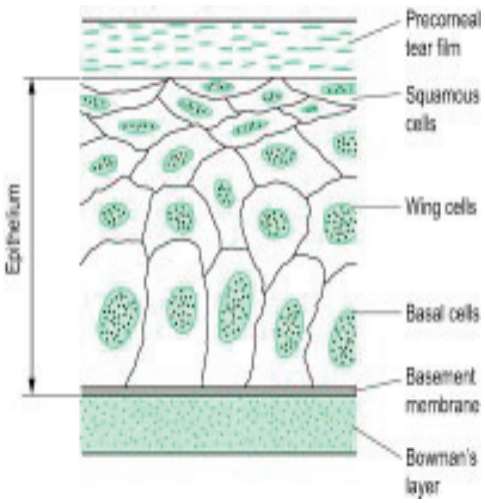


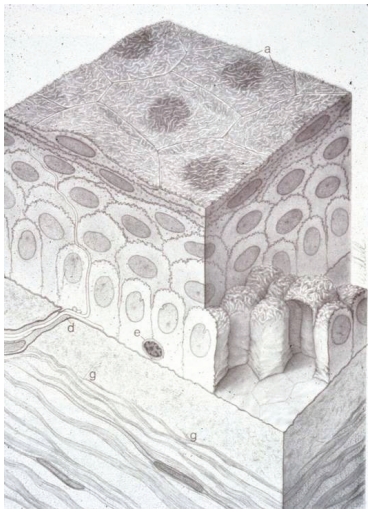
Immune active macrophages derived from the bone marrow are found in the limbal area of the peripheral epithelium. These cells can be recruited to the center of the cornea in response to lesion, inflammation and graft rejection. These Langerhan's cells express human lymphocyte antigens on the surface.

Wing cells are found under the superficial layer. The name of these cells derives from the particular stretched slightly flattened shape. They are held together by tight intercellular junctions.

The basal cells are found beneath and comprise the deepest epithelium corneal layer. This single-cell layered is composed of columnar epithelium that are about 20 μm in height. These cells are capable of mitosis. The only other cells capable of mitosis in the corneal epithelium are the stem cells found in the limbus region in the periphery. The wing and superficial epithelium cells derive from this population of cells. Gap junctions and zonulae adherens line the lateral intercellular junction. They are attached to the underlying basement membrane by an extensive hemidesmosomal system, which aid in preventing epithelium detachment. Recurrent erosion syndrome, persistent epithelium defects, epithelium edema and other clinical manifestations can occur if this bond system is compromised.

The basement membrane, which is about 0.05 μm thick, is formed by an extracellular matrix secreted by the basal cells. Basement membrane regeneration upon lesion requires about a month and a half. During this period, the bond between membrane and overlying epithelium cells and underlying Bowman is fragile and can be disrupted during the healing process. Type IV collagen and laminin are the major components of a healthy basement membrane, however, high levels of fibronectin can be found in response to acute epithelium lesions and inflammation. The basement membrane adheres to the underlying Bowman's membrane with the aid of anchoring fibrils and plaques.





- **Bowman's layer:**

The Bowman's membrane (also known as anterior limiting lamina or anterior elastic lamina) was discovered by Sir William Bowman (1816–1892), an English physician, anatomist and ophthalmologist. It is composed of a smooth layer of irregularly arranged collagen fibers, mainly type I collagen fibrils located between the superficial epithelium and the stroma that helps the cornea maintain its shape and acts as a tough layer that protects the corneal stroma. This layer is about 8-14 μm thick. Scarring and corneal opacities can occur if the Bowman's membrane is damaged.

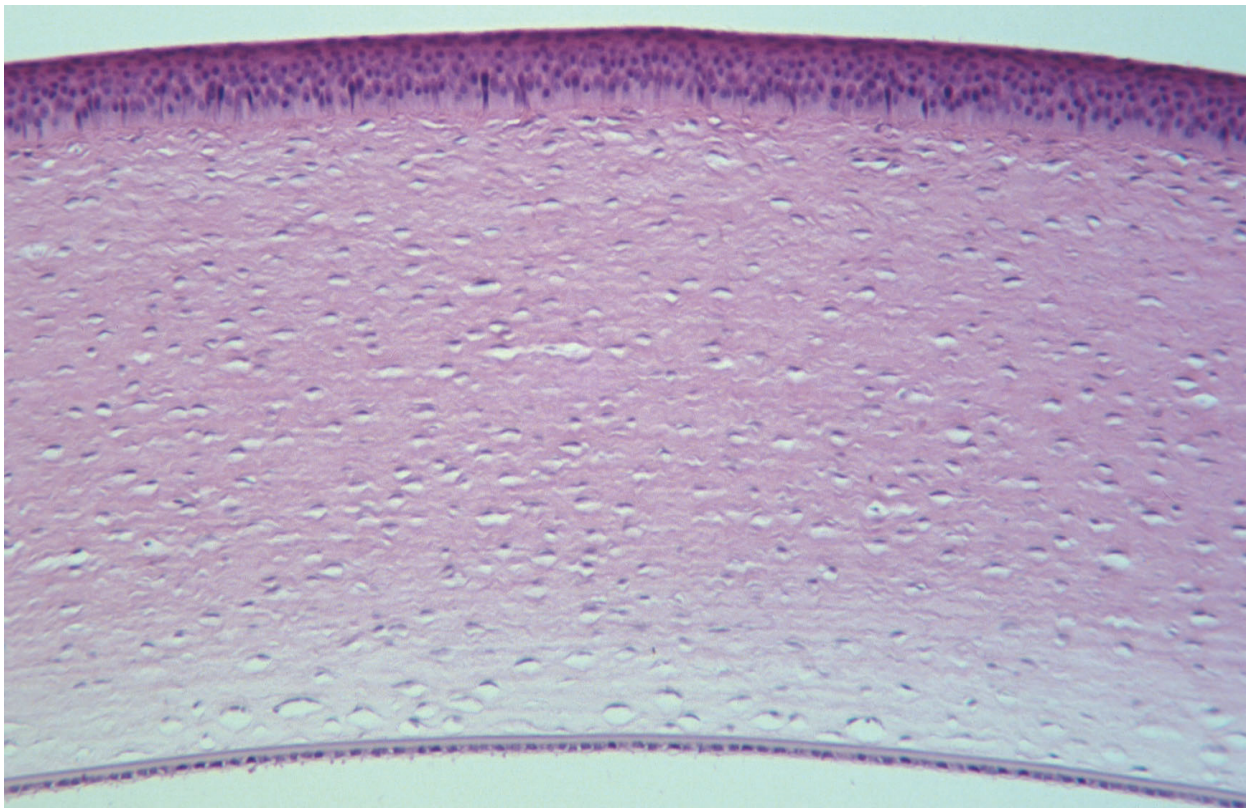
- **Stroma:**

The stroma (or substantia propria) is the thickest layer of the cornea located behind Bowman's layer and in front of Descemet's membrane. It represents approximately 90% of the total corneal thickness and gives the cornea its strength. This thick, transparent middle layer consists of regularly arranged collagen fibers in addition to sparsely distributed interconnected keratocytes, which are the cells needed for general repair and maintenance. The collagen fibers are parallel and superimposed. The corneal stroma consists of numerous layers, composed of about 200-300 lamellae of parallel collagen fibrils in the centre that increase peripherally and reach nearly 500 lamellae at the limbus. Each fibril is about 1.5-2.5μm in size, and composed mainly of type I collagen. The spacing between collagen fibrils in the stroma must be within 200 nm to permit proper stromal architecture and transparency. It is thought that the particular lattice arrangements of the collagen fibrils in the stroma is what provides tissue transparency, due to light scatter by individual fibrils that gets cancelled by destructive interference from the scattered light from other individual fibrils

The stromal collagen fibrils are surrounded by proteoglycans consisting of keratan sulfate and dermatan sulfate (chondroitin sulfate B). These proteoglycans have an important structural function and help regulate hydration. The orientation of the lamellae are different, however, tend to be all parallel to the corneal surface. In the central part of the cornea, the majority of the collagen fibrils are orientated in the inferior-superior and nasal-temporal directions, whereas at the limbus, they are orientated circumferentially, providing greater resistance to forces perpendicular to the axes of the fibrils.

Keratocytes are the predominant cell type found between lamellae of the stroma and play a role in maintaining its organization. These elongated flattened cells from which the collagen fibrils are produced during development, are in contact with the matrix and other cells by long cytoplasmatic extensions. When edema develops in the stroma due to

trauma, disease, inflammation or hypoxia, these cells can be activated and some of the fibrils lose their usual uniform caliber, become displaced and fluid accumulates between the lamellae, causing loss of transparency.



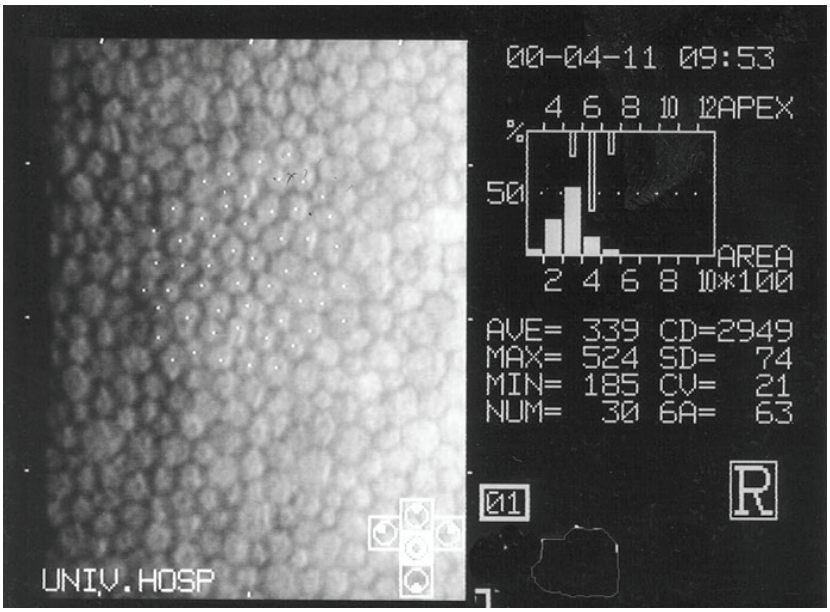
- **Descemet's membrane:**

Also known as posterior limiting membrane, Descemet's membrane separates the endothelium from the stroma. This elastic membrane derived from the endothelium cells, thickens with age and is composed of a thin acellular layer that serves as the basement membrane of the corneal endothelium. It is composed of an anterior layer with a banded appearance and a posterior layer with an amorphous texture. Descemet's membrane is composed mainly of collagen type IV & I fibrils, and is about 5-20μm thick, depending on the subject's age.

- **Endothelium:**

The corneal endothelium consists of a single layer of cuboidal mitochondria-rich cells approximately 5μm thick. The normal healthy cornea endothelium density at birth is about 3500–5000 cells/mm^2, which tends to decrease with age, trauma, inflammation, pathology and surgery. These cells have little or no self-renewing potential. Unlike the corneal epithelium, the cells of the endothelium do not regenerate. Instead, they stretch to compensate for dead cells, causing a reduction of the overall endothelium cell.

One of the main roles of the endothelium is that it is responsible for regulating fluid and solute transport between the aqueous humor and corneal stromal compartments. The endothelium has intracellular and membrane-bound ion transport systems that establish an osmotic gradient from a relatively hypo-osmotic stroma to a hypertonic aqueous. This osmotic gradient allows movement from the stroma to the aqueous to maintain a homeostasis permitting a constant percentage of water in the stroma of about 75%, which is essential for the corneal clarity and transparency. If the endothelium can no longer maintain a proper fluid balance, stromal swelling due to excess fluids and subsequent loss of transparency will occur. This may cause corneal edema and interference with the transparency of the cornea, thereby reducing visual capacity.



2. OCULAR SURFACE WOUND HEALING

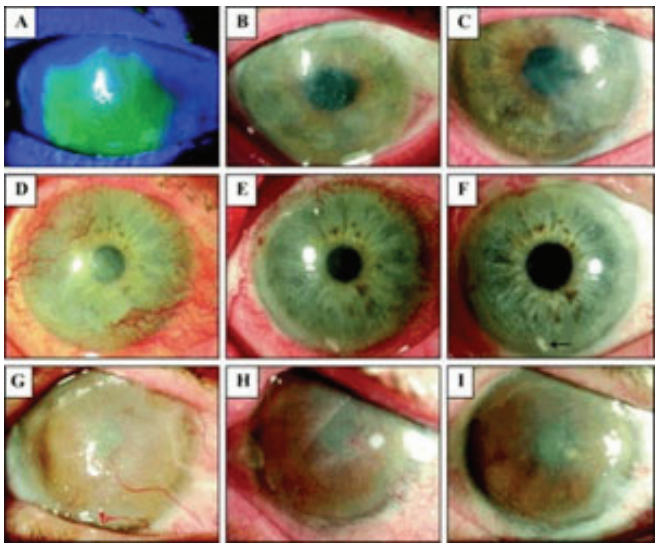
The corneal wound healing response is based on a complex cascade of events that involves cytokine mediated interactions between the different components of the cornea and the immune system. In brief, the cascade of events is brought on by ocular surface damage, which can cause epithelium loss and keratocyte apoptosis and necrosis. This activates early epithelial repair and induces keratocyte proliferation and activation. Some keratocytes undergo myofibroblast differentiation and migration. This is followed by infiltration due to inflammatory response and stromal remodeling. Epithelial surface closure is usually reached. In time, myofibroblasts and inflammatory cells undergo apoptosis and necrosis, and keratocytes gradually return to a normal state over time.

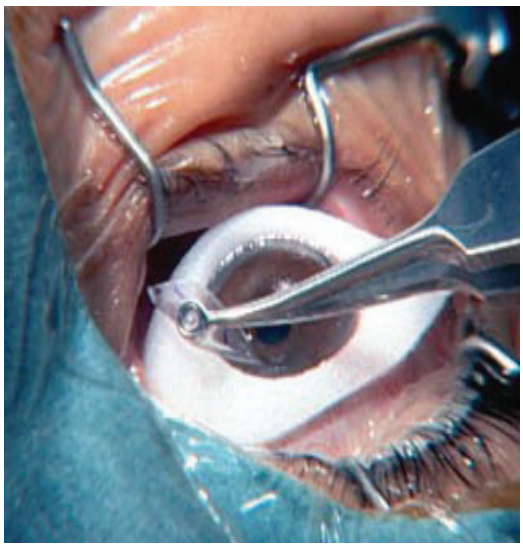
The process of repair commences in response to corneal injury, which initiates quite rapidly within the first hours. The lesion can induce cytokine release from the injured epithelium and epithelial basement membrane. These factors include interleukin (IL)-1 tumor necrosis factor (TNF) alpha, bone morphogenic proteins (BMP) 2 & 4, epidermal growth factor (EGF), platelet derived growth factor (PDGF). Some of these cytokines interact with nearby keratocytes, which induces apoptosis and regulated cell death.

IL-1 is a major cytokine in activating the cascade of events. IL-1 alpha and IL-1 beta mRNAs and proteins are produced in the corneal epithelium, keratocytes and corneal fibroblast. Immunocytochemistry studies have shown that there are no signs of IL-1 alpha or beta in keratocytes of healthily corneas, and exposure to IL-1 can induce keratocytes to produce IL-1 by an autocrine mechanism. IL-1 protein can be detected in apoptotic keratocytes or myofibroblasts and in tears of eyes with ocular surface damage, inflammation, infection and severe dry eye syndrome. If the epithelium and basal membrane are intact, the cytokine cannot interact and bind to stromal keratocyte IL-1 receptors to activate these cells. IL-1 also regulates hepatocyte growth factor (HGF) and

keratinocyte growth factor (KGF). HGF and KGF are produced by activated keratocytes and myofibroblasts and are involved in the proliferation, motility, and the differentiation of epithelial cells. IL-1 also upregulates the expression of enzymes by keratocytes, such as collagenases and metalloproteinases, which cause remodeling of collagen during corneal wound healing. IL-1 and TNF alpha induce keratocytes and corneal epithelial cells to produce chemokines such as IL-8, monocyte chemotactic protein (MCP)-1. PDGF is another important cytokine released by the epithelial cells and involved in the early phases of wound healing. PDGF modulates corneal fibroblast proliferation, chemotaxis, and differentiation.

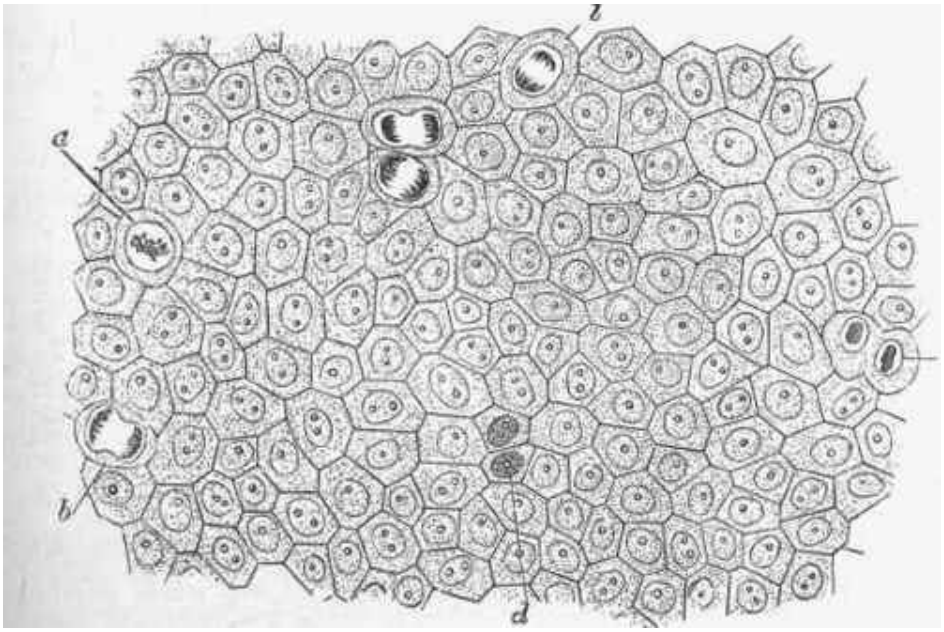
The cascade of lesion-induced cytokines initiate early stromal keratocyte apoptosis and necrosis, which can continue for 7-10 days after trauma. A variety of epithelial injuries can induce this process, which include mechanical de-epithelization, iatrogenic corneal surgical procedures, infection, trauma, and chemical burns. Apoptosis can be identified with immunohistochemical preparations using TdT-mediated dUTP nick end labeling (TUNEL assay).





Growth factors that modulate epithelial healing like HGF and EGF tend to increase in the lacrimal gland, which could serve as an additional source of cytokine production that regulate proliferation, migration and differentiation during the early wound healing. Epithelial cells and exposed keratocytes can be influenced by cytokines in the tear film derived from the lacrimal gland, the conjunctiva or even the conjunctival vessels.

During the early phases of epithelial healing that occur within the first few days, the various growth factors and the cytokines begin to induce new basement membrane formation and cause surface epithelium to replicate, migrate and gradually cover the exposed ocular surface. The epithelial stem cells located at the limbus begin move from the periphery in a sideways fashion, together with the basal cells that move vertically to the apical layers. This follows the same pattern of normal epithelium turnover.



The continual apoptosis , necrosis and pro-inflammatory process cause activation of residing stromal keratocytes, which begin to proliferate, migrate and induce myofibroblast formation. Myofibroblasts, presumed derivatives of keratocytes after induction with TGF-beta, have contractile alpha-smooth muscle actin and can be seen under the basement

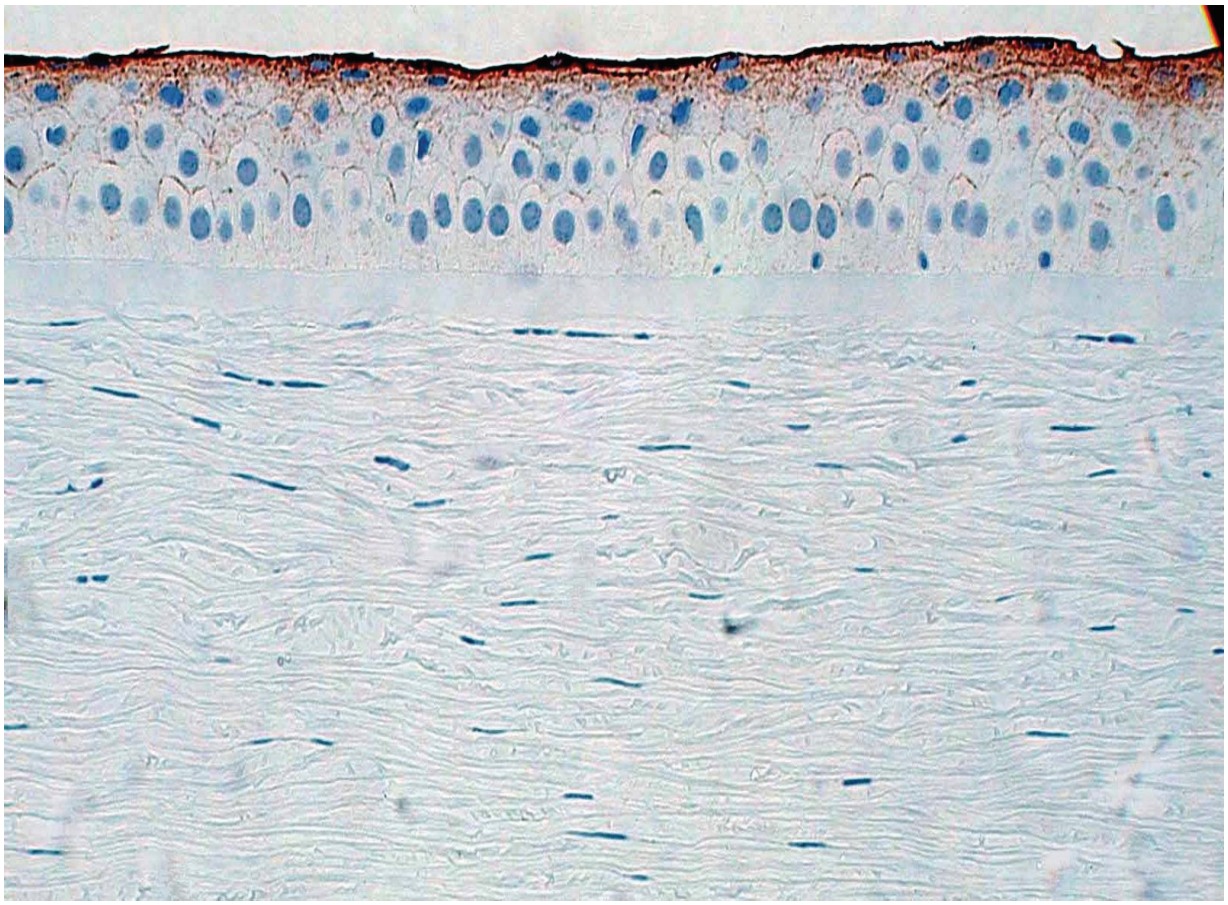
membrane in the anterior stroma even weeks after lesion. Activation of these fibroblasts can cause reduced stromal transparency and corneal haze due to collagen and extracellular matrix remodeling through production of collagen, glycosaminoglycans, collagenases, gelatinases and matrix metalloproteinases (MMP). Myofibroblast cytokine TGF-β is also involved in stimulating corneal scarring, by binding to specific cell surface protein receptors.

Inflammatory cell infiltration is an important process induced soon after lesion. IL-1 and TNF-alpha on the epithelium and keratocytes triggers cytokine release and stromal infiltration by macrophages, monocytes, T cells and polymorphonuclear cells. These inflammatory cells arrive from peripheral blood vessels and tear film. Studies have shown that fibroblasts produce monocyte chemotactic and activating factor (MCAF) in vitro when stimulated by IL-1 or TNF.

The stroma undergoes remodeling during the phases of wound healing and repair. The cascade of events cause a breakdown and synthesis of collagen and other components of the stroma. This cross-link between collagen fibers gradually develop to stabilize the lesion in the months following injury. Newly formed collagen fibers do not follow the normal stroma architecture, in addition to the presence of abnormal extra cellular matrix (ECM) proteoglycans and collagen types. Remodeling over months and years transforms the repaired tissue to approximate normal corneal organogenesis. The altered architecture and components that make up the remodeled cornea renders it less transparent, which may cause permanent corneal scarring. Culture studies have shown that the cells that produce the collagenases during the remodeling phases are fibroblasts, PMNs and macrophages. The parallel layers of collagen lamellae normally found in healthy corneas begin to reform over across the injured region. Electron microscopic analysis show that collagen fibril progressively become more regular in size and arrangement. During these phases of remodeling, matrix degrading enzymes (MMP) play

a key role in degrading abnormal ECM components formed after wound healing. They are produced by resident and invading inflammatory cells, and are secreted in the form of inactive proenzyme that need to be activated. Fibroblast collagenase, for example, is involved in the degradation of native types collagen I, II & III. Stromalysin can specifically cleave proteoglycans, fibronectin and laminin.

Inflammatory, myofibroblast and fibroblast cells gradually leave the cornea or undergo apoptosis and keratocytes return to a quiescent state. Complete wound healing with resulting corneal transparency is possible if the trauma is not too severe and if all the factors and mechanisms involved in this cascade of events function properly.



3. ADIPOSE TISSUE

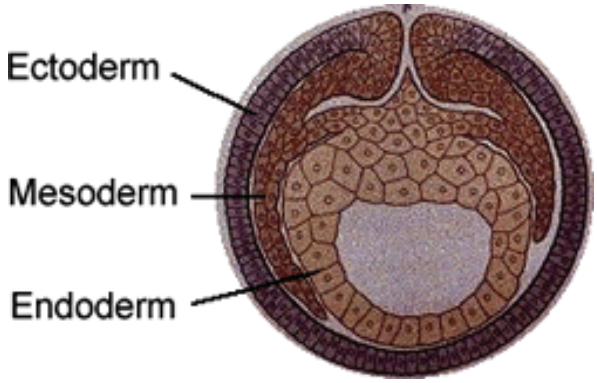
- **Embryology**

During the first month of pregnancy, the developing embryo starts to grow and take on a form that will determine the complete formation of the entire body. The differentiation process begins in the first month after the spermatozoa has fertilized the egg, and rapidly progresses during the fetal period. Organogenesis, which involves a series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo, gives rise to three germ layers from which all the bodily organs and tissues then develop:

1. **Ectoderm**, which differentiates into epidermis, hair, nails, tooth enamel, glands, epithelia of the mouth, parotid gland, nasal and tear duct epithelia, cornea and conjunctiva epithelium, various parts of the nervous system (spine, peripheral nerves and brain), lymph nodes, and blood components.

2. **Mesoderm** that forms mesenchyme (connective tissue), mesothelium, non-epithelial blood corpuscles and coelomocytes. The tissues that arise from this layer include skeleton of the body, connective tissue of the gut, peritoneum, derma, adipose tissue, kidneys, urinary system, gonads (testicles and ovaries), reproductive organs, heart, vessels, and muscle.

3. **Endoderm** gives rise to the gastrointestinal tract, liver, pancreas, endocrine glands, respiratory tract, thyroid, prostate, urethra, trachea, larynx, spermatozoa and oocyte.



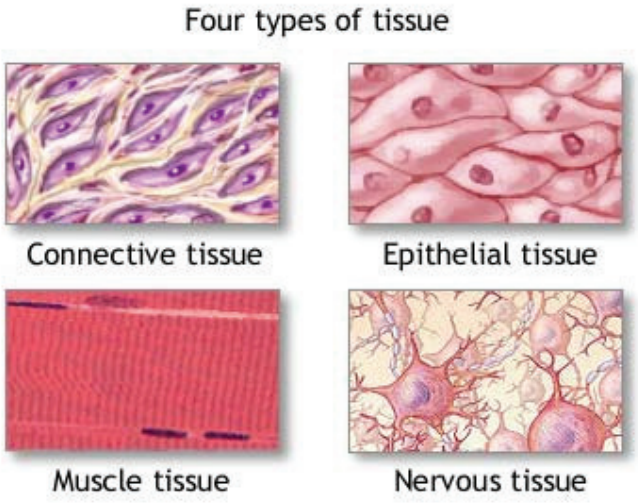
The complexity and variety of cellular expression derived from these three layers are due to the activation and expression of different genes in cells that derive from the same genome. Specific genetic expression, which is dependent on intracellular and extracellular factors, gives rise to the production of specific proteins. During embryonic development, cells take on a specific cellular line, thus become committed to differentiate and produce specific proteins. Once committed, most cells continue toward a specific lineage to develop into one of the four main tissues that make up the human body, which include:

I. **Epithelial tissue**: characterized by the presence of polyhedral cells distributed in layers that are tightly in contact with each other, which form mechanical and functional intercellular complexes junctions. Moreover, these cells are in close contact with an epithelium-connective sheet known as the basal lamina. Epithelial tissue is usually free of capillaries, however, have the capacity to proliferate.

II. **Connective tissue**: contains abundant extracellular fluid and fibers between cells. This tissue is highly vascularised and has a high capacity to proliferate.

III. **Muscle tissue**: formed by cells containing microfilaments that are highly in contact with each other and have contractile ability.

IV. **Nervous tissue**: made of cells with characteristic prolonged cytoplasm, thus permitting morphological and functional intertwining connections between nerve cells, glia and muscle.



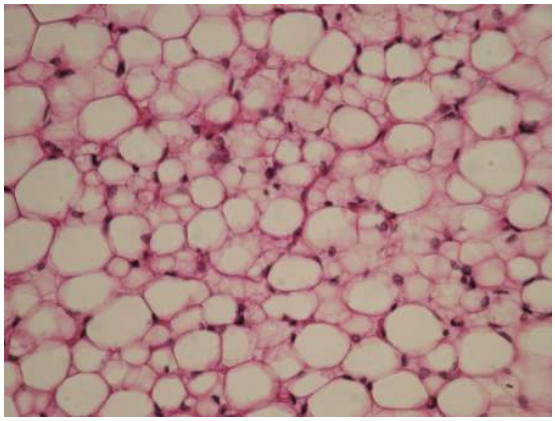
- **Characteristics**

Adipose tissue is a specialized loose connective mesoderm-derived tissue composed of adipocytes. The main function of this tissue includes the major storage site for fat in the form of triglycerides. There are two different forms of adipose tissue found in mammals; white and brown adipose tissue. The presence, amount, and distribution vary in different species and individuals.

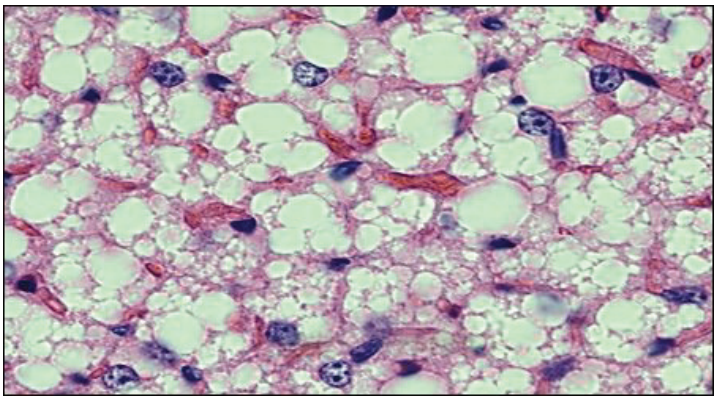
White adipose tissue (or sometimes considered as yellow) serves three functions: heat insulation, mechanical protection, and most importantly, source of energy. About 50% of the distribution can be found in the form of subcutaneous adipose tissue, located directly below the skin. It is an especially important heat insulator in the body, which is dependent

upon the thickness of this fat layer. About 45% surrounds internal organs, providing a cushioning protection during abrupt and harsh movements. The remaining 5% is in contact with muscle tissue. As the major reservoir of storage, fat provides energy. It is an efficient way to store excess energy, especially considering it contains very little water; thus more energy can be derived from a gram of fat than from a gram made up of carbohydrates or proteins.

The primary function of white fat tissue is to store triglyceride in periods of energy excess and to release energy in the form of free fatty acids when needed. Fat tissue also plays an important role in numerous processes through its secretory products and endocrine functions, which play a role in immunological responses, vascular diseases and appetite regulation. Leptine is a peptide hormone primarily made and secreted by mature adipocytes, which effects appetite, body weight regulation, fertility, reproduction and hematopoiesis. Adipose tissue is also an important site for estrogen biosynthesis and steroid hormone storage. In addition, this tissue secretes a variety of peptides, cytokines and complement factors, which act in an autocrine and paracrine manner to regulate adipocyte metabolism and growth, as well as endocrine signals to regulate energy homeostasis.



Brown adipose tissue can rarely be found in human adults. It is normally present in new-born children. It is also found in small animals and hibernating mammals. The darker color is due to the rich vascularisation and densely packed mitochondria. In non-hibernating animals, brown adipose tissue is metabolically less active, although cold exposure can activate it. In hibernating animals and human new-born babies, it plays an important role in regulating body temperature and thermogenesis. Brown adipose tissue can directly release energy in the form of heat due to the metabolism of the mitochondria.

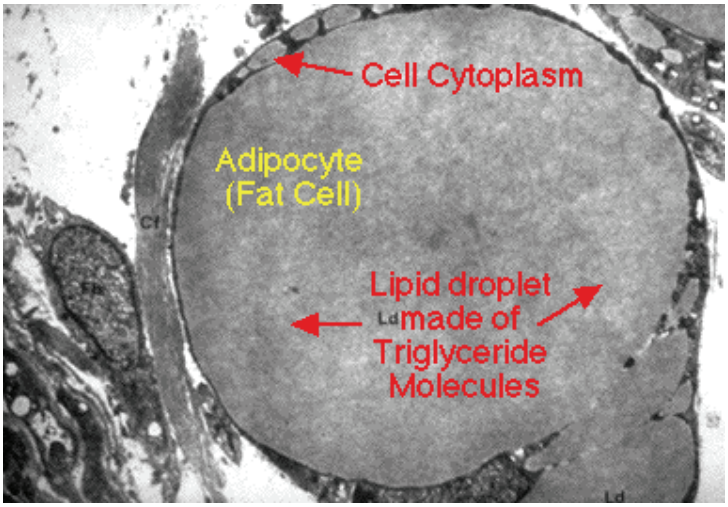


- **Cytology**

White adipose tissue is composed mostly of lipid-filled cells called adipocytes, which are found amongst collagen fibers. Adipose tissue also contains stromal-vascular cells including fibroblastic connective tissue cells, leukocytes, macrophages, and pre-adipocytes. The adipose cell appears as a round ring structure that encloses a large lipid drop with a peripherally displaced nucleus. These unilocular cells range in size from 25 to 200 microns. This large lipid droplet does not appear to contain any intracellular organelles. Mitochondria can be found near the nucleus. The cell cytoplasm contains a small amount of rough endoplasmic reticulum, very discrete amounts of smooth endoplasmic reticulum, and numerous ribosomes. The Golgi apparatus and mitochondria are not highly represented. Electron microscope analysis shows that the external surface

is covered with reticular fibers composed of glycoprotein, which is in close contact with the adipose vascular system. Each adipocyte in the white adipose tissue is in contact with at least one capillary. This blood supply provides sufficient support for the active metabolism, which occurs in the thin rim of cytoplasm surrounding the lipid droplet.

Approximately 60 to 80% of the weight of white adipose tissue is lipid, mostly composed of triglyceride. Small amounts of free fatty acids, diglyceride, cholesterol, phospholipid, cholesterol ester and monoglyceride are also present. The fatty acids that make up the lipid component include myristic, plamitic, palmitoleic, stearic, oleic, and linoleic acid. The remaining weight of white adipose tissue is composed of water, which makes up about 5 to 25% and trace amounts of protein that make up about 2 to 3%.



- **Lineage and differentiation**

Adipocytes are considered to originate from fibroblast-like precursor cells, in which differentiation is influenced by appropriate stimulatory conditions. The criteria used to identify adipocytes include the intracellular capability of lipid accumulation. Studies suggest that the adipocyte tissue derives from embryonic stem cell precursors that have the capacity to differentiate into the mesodermal cell types, which include differentiation

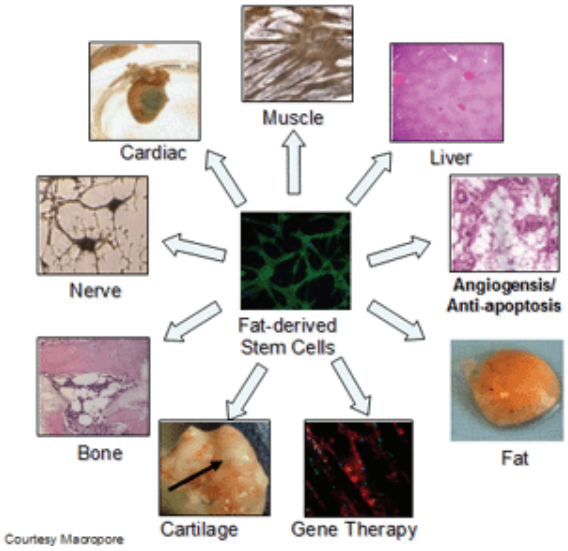
into adipocytes, chondrocytes, osteoblasts, and myocytes. Adipoblasts, which are the earliest unipotential cells, can commit to the adipogenic lineage and become preadipocytes. These cells can differentiate into mature adipocytes that are able to synthesize and store lipids when placed in the proper environmental conditions. The early molecular events that promote mesenchymal precursor cells to the adipogenic lineage are not well known. The expansion of white adipose tissue throughout the body is due to an increase in adipocyte cell size and number.

During tissue development, committed preadipocytes withdraw from the cell cycle, stop proliferating and undergo adipocyte differentiation upon reaching confluence. Adipocyte differentiation is characterized by the selective expression of specific genes, which occur mainly at the transcription level that give rise to the specific adipocyte phenotype. Several hormones and growth factors have shown to play a role in adipocyte differentiation by activation of specific receptors, which mediate differentiation signals through a cascade of intracellular events. Insulin like growth factors have been shown to be essential regulators of fat cell formation, differentiation and growth. Glucocorticoids have been used in numerous studies to induce differentiation of cultured preadipocyte cell lines and primary preadipocytes. Dexamethasone, for example, is believed to operate through activation of a nuclear glucocorticoid receptor that regulates transcription. Estrogen and progesterone have an effect on preadipocyte replication in vitro and adipogenesis. Retinoic acid has shown to influence differentiation and maturation of preadipocytes. Growth hormones and thyroid hormone seem to accelerate the differentiation into mature adipocytes.

- **Adipose derived stem cells (ADSC)**

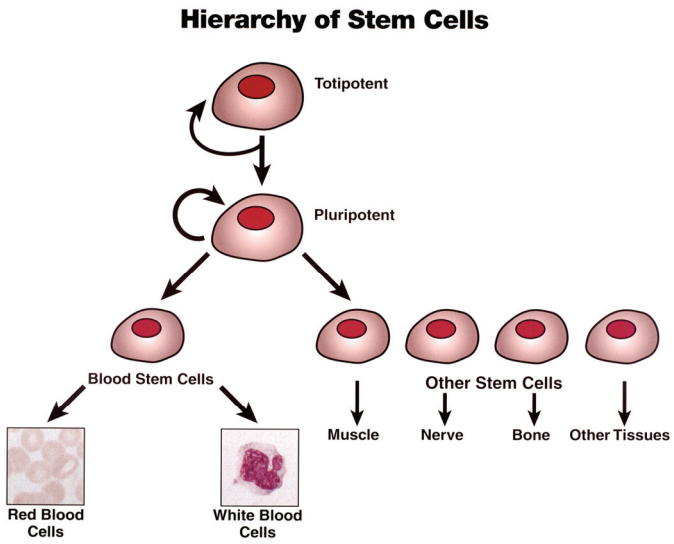
In the past several years, numerous studies have shown the existence of multipotent adult progenitor cells in various tissues and locations throughout the body, such as skeletal muscle, bone marrow, synovial tissue, and periostium. In the

past ten years, studies have reported that human adipose tissue also contains a population of multipotent stem cells, which can be isolated in large amounts from liposuction or biopsy, and expanded in culture. When exposed to the proper growth conditions in vitro, these adipose derived stem cells (ADSC) can successfully differentiate into various mesenchymal cell lineage, which include adipogenic, osteogenic, angiogenic, chondrogenic, myogenic, cardiomyogenic, and neurogenic.



In general, stem cells are very different from other mature cells found throughout the body, in that the show endless capacity of cell multiplication. When stem cells divide, the daughter cells maintain the same potential of the mother cells, thus the stem cell depot can be conserved in the tissue without going into depletion. These cells can give rise to numerous cell copies, which in turn can take on a distinct cell differentiation according to micro-environmental factors. Another important aspect is that these calls are multipotent, thus can be induced to differentiate into cells that are of different origin. By using different culture mediums, hormones and growth

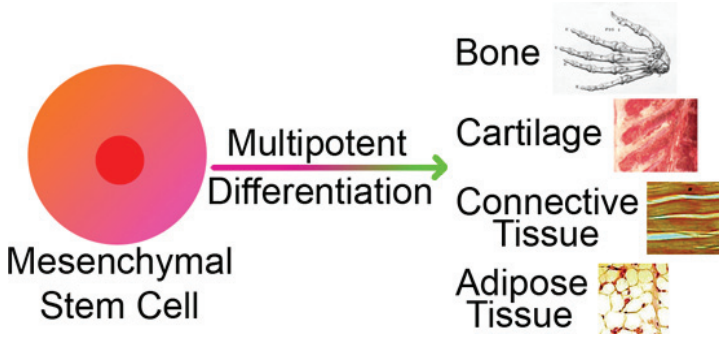
factors, these cells can be conditioned to form different cells lines in vitro. Although stem cells have similar characteristics, various types of stem cells from varying origin can be found in different organs; these cells have different phenotypes that produce specific proteins and can thus be distinguished by specific biochemical markers.



The precise lineage of adipose tissue is not completely know, however, like muscle and bone, it is generally regarded that adipose tissue derives from a mesodermal origin. Studies have reported that initial origins can be traced back to the neural crest phases during embryologic development, thus from both a neurectoderm and mesoderm descent. Histological studies on primitive organ development have shown that early clusters of adipocytes emerge from bulks of mesenchymal cells related to the development of the local blood system network, thus vascularisation plays an important role in adipose tissue formation. Angiogenesis and adipogenesis appear to be coordinated in space and time.

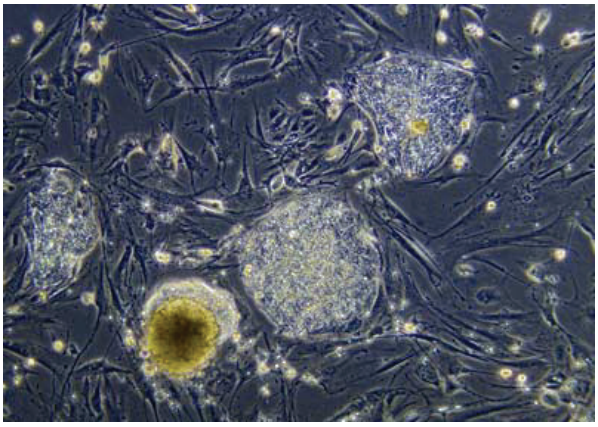
Adipose precursor cells in fact express surface markers that are also found on immature endothelial cells. Some authors have hypothesized that ADSC could differentiate from a variety of sources, including various vascular cell types found within the white adipose tissue, but this issue still remains debatable.

The cell surface phenotypes of ADSC are quite similar to bone marrow-derived mesenchymal stem cells, in that both cell types express similar mesenchymal stem cell related surface markers. These distinct cells, however, exhibit unique characteristics and differences in gene expression. In vitro studies have also shown that under osteogenic conditions, ADSC can express genes and proteins associated with an osteoblast phenotype. In vitro differentiation studies have also seen this to be true for neuronal lineage, myoblasts, cardiomyocytes, hematopoietic cells, and corneal keratocytes.

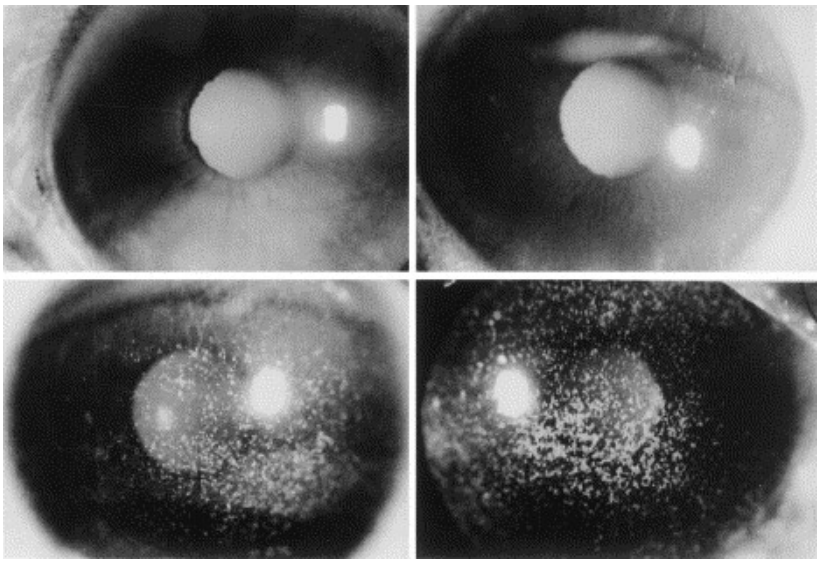


For many years, bone marrow derived stem cells have been considered as the prime source of mesenchymal stem cells for clinical applicability for cell-based therapies and tissue engineering purposes. Over the past several years, however, autologous ADST derived from lipoaspirate have shown to be an advantageous source of stem cells considering they can be readily harvested in large numbers with low donor morbidity. These mesenchymal cells exhibit the ability of self-

renewal and ability to differentiate in multiple lineages. Unlike stem cells retrieved from bone marrow, umbilical cord blood, peripheral blood and other tissues, adipose tissue serves as a large reservoir of mesenchymal stem cells that have shown to be 100 fold more numerous in comparison to other autologous sources.



Numerous studies and clinical trials in the past decade have reported the safety, efficacy and therapeutic advantages in clinical applications, which can be found in all areas of medicine including plastic surgery, orthopedic surgery, cardiology, oral and maxillofacial surgery, cardiology, and neurological applications. Current scientific literature has just begun to report the therapeutic applications of ADSC in ophthalmology, and the clinical applications in this field look promising.



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5. INTRODUCTION AND AIM OF THE EXPERIMENTAL STUDY

The cornea provides a protective barrier and clear optical pathway for the visual system, representing two thirds of the eye's refractive power.[1] This avascular and transparent structure is composed of three layers, which include the outer most non-keratinized stratified epithelium, stroma and single-layered endothelium. The corneal epithelium is about 5 to 7 layers thick and makes up the anterior ocular surface together with the conjunctiva, which are both constantly lubricated with the tear film that provides adequate trophism and antibacterial protection. The thin layered endothelium lies behind the Descemet membrane and is in contact with the aqueous humor of the anterior chamber with active pumping functions that maintain corneal homeostasis. The stroma accounts for about 90% of the cornea and can reach up to 500 micron in humans. It is composed of dense collagenous tissue with keratocytes, which are normally quiescent and are responsible for secreting the extracellular matrix. Most corneal diseases and trauma involve the epithelium and stroma, and include immune disorders, chronic inflammation, infection, iatrogenic procedures (i.e. laser refractive surgery), corneal dystrophies, mechanical and chemical injuries, limbal stem cell deficiency and ectatic diseases. These ocular disorders can lead to severe inflammation, Stevens-Johnson syndrome, persistent epithelium defects, neovascularization, conjunctivalization, persistent corneal opacities and scarring, which all lead to permanent vision loss.[2, 3]

The stroma is one of the most important layers involved in corneal wound healing. It provides strength and refractive power, providing a transparent optical medium due to the parallel aligned collagen fibrils composed of collagen types I and V, which are associated with several proteoglycans (i.e. keratocan, lumican, and mimecan) that make up the stromal extracellular matrix (ECM) and cells that secrete them, mainly keratocytes.[1, 4, 5] These principle cells of the stroma are flat and dendritic in morphology, are positive for

markers (i.e. CD34 and aldehyde-3-dehydrogenase or ALDH), and produce collagen, keratin sulfate proteoglycans (KSPK), and other ECM factors that can be used as specific keratocyte-specific markers.[6] These mesenchymal derived cells that make up the stroma are normally mitotically quiescent after embryonic development; collagen synthesis decreases in adults while the synthesis of KSPK remains high, and the homeostasis of these ECM components provides proper fibril and ECM architecture and corneal transparency.[5] Studies have shown that loss of stromal transparency is related to reduced KSPK and ALDH.[7-9] Trauma, burns, inflammation, iatrogenic procedures, infections and ocular pathologies activate stromal wound healing, which induces keratocytes to lose their dendritic morphology and differentiate into fibroblasts and/or myofibroblasts. These activated cells secrete altered ECM components with reduced KSPK, which disrupt the collagen fibril and ECM architecture, resulting in reduced transparency and stromal scarring.[10] The degree of lesion determines how the cornea heals, which is why mild stromal damage can lead to new collagen fibril and ECM production while still allowing transparency to be maintained. Severe corneal injuries and several ocular pathologies can induce altered repairing mechanisms, which cause permanent scaring and infiltration due to inflammation that can also give rise to ocular surface tissue and keratocyte loss.[1]

Corneal opacification is the second major cause of blindness worldwide after cataract, which affect more than 8 million individuals.[11] The most common intervention for restoring corneal clarity and function is full thickness penetrating or lamellar keratoplasty, however, there are numerous limits that include scarce donor-quality tissue, elevated surgical costs, graft failure and immune rejection.[5] Alternative surgical techniques include amniotic membrane transplantation and conjunctival limbal autograft from the healthy fellow eye, but surgical outcomes and limitations are similar to keratoplasty.[1] Non surgical medical treatments are usually geared towards inhibition of inflammation and moderating the cascade of events related to an active immune response. Alternative topical methods

that are currently being used in clinics have shown to enhance ocular surface wound healing, and include autologous serum[12] and autologous plasma rich in growth factors.[13, 14] These eye drops made from the patient's whole blood are rich in nutrients, growth factors, vitamins and neuropeptide, which provide epitheliotrophic, antimicrobial and anti-inflammatory factors that are thought to be responsible for the therapeutic effect. Studies have reported that autologous drops seem to improve corneal epithelial healing in patients with severe dry eyes, recurrent erosion syndrome, persistent epithelial defects, chronic inflammation, and adjunctive treatment in ocular surface reconstruction,[12-14] however, there is limited data regarding their wound healing effects on stromal scarring and damage.

The use of stem cells in regenerative medicine has generated considerable interest in the past years. Friedenstein was the first to report fibroblast shaped cells in bone marrow that were capable of differentiating into various lineages of the mesoderm in the late 1960's and named them mesenchymal stem cells (MSC).[15] It was later discovered that MSC cell populations with similar properties reside in almost all human tissues.[16] Studies by Zuck et al were the first to report a MSC population with similar characteristics to those found in the bone marrow from human adipose tissue.[17] These adipose derived stem cells (ADSC) were shown to have the capability of differentiating into multiple mesodermal cell type, had clonogenicity properties, were pluripotent, capable of lineage-specific activity and expressed multiple CD marker antigens and proteins similar to those observed on bone marrow MSC.[17, 18] The interest in ADSC has greatly increased considering that it is easy to obtain large quantities of autologous tissue in a rapid minimally invasive liposuction procedure that requires only local anesthesia. Moreover, ADSC are 100 times more abundant in adipose tissue than MSC in bone marrow.[19] Numerous clinical trials in the past decade have reported the use of ADSC to treat acute and chronic diseases

afflicting various organs and tissues, including bone, heart, central nervous system, gastrointestinal tract, bone marrow, liver, pancreas and skin.[20-22]

Arnalich-Montiel et al were the first to report the successful use of ADSC obtained from processed human lipoaspirate for corneal stroma repair in a rabbit model.[6] Human ADSC were delivered to a surgical pocket made 50 microns deep within the stroma. The transplanted cells were shown to be safe, non-immunogenic, preserved corneal transparency and able to differentiate into functional keratocytes that produced type I & VI collagens and expressed ALDH and cornea-specific proteoglycan keratocan. Other studies have shown the inhibition of inflammation and angiogenesis in treating chemically burned rat corneas with MSC obtained from human bone marrow[3] and rat primary cell lines.[2]

Surgical techniques can be applied to potentially deliver ADSC to the corneal stroma, however, this may induce further iatrogenic damage to an already injured cornea and the procedure does not seem feasible or cost-effective. Studies regarding tissue engineering and scaffolds are ongoing, yet still not clinically applicable. The purpose of our study was to assess the topical use of ADSC derived from human processed lipoaspirate in treating ocular surface damage. The study included data from experiments performed from 2009 to 2011 in Udine and Calabria (Italy) and Oviedo (Spain). Cornea lesion experiments and histological assessments were first performed prior to establishing the best chemical burn model to be used in our wound healing study. Rats underwent chemical burn lesions and randomly assigned to different treatment groups. Additional experiments were performed in mice eyes using a laser induced corneal lesion model. Clinical and histological assessments were compared between groups to determine biosafety, immunogenicity and efficacy of human derived ADSC in treating induced epithelial and stromal wounds in animal corneas.

6. MATERIAL AND METHODS

Animals

Nineteen male Albino Wistar rats (280–330 g) and 40 black mice C57BL/6 (30-40 g) were used in the experiments. Animals were housed with a 12-h light–dark cycle with ad libitum access to food and water. Animal care and experiments were carried out in accordance with the guidelines of the Italian Ministry of Health for Animal care (DM 116/1992) and the Association for Research in Vision and Ophthalmology Research on the Use of Animals in Ophthalmology and Vision Research and approved by the Institutional Animal Care and Use Committee.

Prior to induction of lesion and treatments, animals were anesthetized by intraperitoneal injection of 10 mg/kg zolazepam and 10 mg/kg xylazine hydrochloride. Topical anesthesia was induced by 0.4% oxibuprocain eye drops (Novesina, Novartis, Varese, Italy). For the rat experiments, 6 animals were sacrificed by cervical dislocation after induction of the chemical lesion to assess histology of corneal damage, while the remaining 13 animals were sacrificed at 74 hours after induction of the ocular surface lesion. For the mice model, 16 of the 33 animals were sacrificed on day 3, while the remaining 17 on day 8. All animal eyes were enucleated and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature, then rinsed and stored in PBS at 4°C until further processing.

Isolation and preparation of adipose derived stem cells

Human subcutaneous abdominal adipose tissue was obtained from healthy patients (aged 27-62 years) undergoing elective lipoaspiration surgery with informed oral and written consent under a protocol approved by the Institutional Review Board (IRB) of the University of Udine, in accordance with the guidelines of the Tenets of the Declaration of

Helsinki. Patients were screened and resulted negative for HIV, hepatitis B and C virus, and syphilis.

ADSC were obtained from the stromal vascular fraction obtained from lipoaspirates and cultured as previously described.[23, 24] Briefly, the stromal vascular fraction (SVF) was obtained centrifuging the lipoaspirates at 3,000g for 3 minutes. The SVF was subsequently dissociated in Jocklik modified Eagle's medium (JMEM; Sigma-Aldrich, St. Louis, MO) containing 400 U/mL of collagenase type 2 (Sigma-Aldrich, St. Louis, MO) for 20 minutes at 37°C. The collagenase enzymatic activity was stopped with the addition of 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in JMEM. Samples were centrifuged for 10 minutes at 600 g, then pellets were resuspended and filtered through a 40-μm pore-sized membrane. Filtered cells ($2x10^6$ cells per dish) were plated into 100-mm human fibronectin (Sigma-Aldrich, St. Louis, MO) coated dishes in expansion medium composed as follow:[23] 60% low glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA), 40% MCDB-201(Sigma-Aldrich, St. Louis, MO), 1 mg/mL linoleic acid-BSA, 10^{-9} mol/L dexamethasone (MP Biomedicals, Solon, OH), 10^{-4} M ascorbic acid-2 phosphate (Sigma-Aldrich, St. Louis, MO), 1Xinsulin-transferrin-sodium selenite (Sigma-Aldrich, St. Louis, MO), 2% fetal bovine serum (FBS; StemCell Technologies, Vancouver, Canada), 10 ng/mL of human platelet-derived growth factor-bb and10 ng/mL of human epidermal growth factor (both from Peprotech EC, London, UK). Colonies developed in primary culture and reached near confluency in approximately 1 week. Medium was replaced every 3-4 days. Cells were detached with 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) and replated at a density of $2x10^3/cm^2$ once reached at 70-80% confluence. Adherent cells obtained after the second subculture, which corresponds to the third passage of cells, were used for the experiment.

Cells were isolated by the use of the selective medium. Stemness of these cells was demonstrated in vitro in accordance to our previous study [23, 24] on the basis of:

mesenchymal stem cell like surface immunophenotype; expression of Oct-4, Nanog and Sox2 proteins and multipotency, shown by the ability to differentiate into derivatives of all three germ layers.

Flow cytometry

Proliferating cells were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and, after a 20 minutes recovery phase, were incubated with either properly conjugated primary antibodies: CD10, CD13, CD29, CD49a, CD49b, CD49d, CD90, CD73, CD44, CD59, CD45, CD271, CD34, (BD Biosciences), CD105, KDR, CD66e (Serotech), CD133 (Miltenyi Biotec), E-cadherin (Santa Cruz Biotechnology), ABCG-2 (Chemicon International). Properly conjugated isotype matched antibodies were used as a negative control. The analysis was performed by CyAn (Dako Cytomation).

Immunofluorescence

Cells cultured either in expansion or in differentiation medium were fixed in 4% buffered paraformaldehyde for 20 minutes at room temperature (R.T.). For intracellular stainings, fixed cells were permeabilized for 8 minutes at R.T. with 0.1% Triton X-100 (Sigma-Aldrich) before exposing them to primary antibodies. In order to block unspecific binding of the primary antibodies, cells were incubated with 10% donkey serum in PBS for 30 min. Primary antibody incubation was performed over-night at 4°C using following dilutions: Oct-4 (Abcam, 1:150); Sox-2 (Chemicon, 1:150); Nanog (Abcam, 1:150); Cytokeratins 7, 8, 18, 19 (Biogenex, 1:20); ß3-tubulin (Abcam, 1:1000); Smooth Muscle Actin (Dako, 1:50), Connexin 43 (Santa Cruz, 1:40); α-Sarcomeric Actin (Sigma, 1:100), and Gata4 (Santa Cruz, 1:100). To detect primary antibodies, A488 and A555 dyes labeled secondary antibodies, diluted 1:800, were employed (Molecular Probe, Invitrogen). 0.1 µg/ml DAPI (Sigma) was used to identify nuclei. Vectashield (Vector) was used as mounting medium.

Epifluorescence images were obtained utilizing a live cell imaging dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (Leica Microsystems, Wetzlar, Germany).

Multilineage differentiation

Hepatocytic differentiation was induced growing cells for two weeks at high density onto fibronectin coated dishes in a medium containing 0.5% FBS, 10 ng/ml FGF-4 and 20 ng/ml HGF (both from Peprotech EC, London, UK). After this period, FGF-4 and HGF were substituted for 20 ng/ml OncostatinM for another 14 days (Peprotech EC, London, UK). Muscle cell differentiation was achieved plating 0.5 to 1x104/cm^2 cells in expansion medium containing 5% FCS (Sigma-Aldrich, st. Louis, MO, USA), 10 ng/mL bFGF, 10 ng/mL VEGF, and 10 ng/mL IGF-1 (all from Peprotech EC, London, UK), but not EGF. Cells were allowed to become confluent and cultured for up to 4 weeks with medium exchanges every 4 days. For neurogenic differentiation, cells were plated in DMEM-high glucose (Invitrogen, Carlsbad, CA, USA), 10% FBS (Sigma-Aldrich, st. Louis, MO, USA). After 24 h medium was replaced with DMEM-high glucose, 10% FBS containing B27 (Invitrogen, Carlsbad, CA, USA), 10 ng/ml EGF and 20 ng/ml bFGF (both from Peprotech EC, London, UK). After 5 days, cells were washed and incubated with DMEM containing 5 g/ml insulin, 200 □M indomethacin and 0.5 mM IBMX (all from Sigma-Aldrich, St. Loius, MO, USA), in the absence of FBS for 5-10 days. At the end of every treatment, cells were fixed either with 4% buffered paraformaldehyde.

Blood serum

Human serum was prepared in accordance to the Azienda Ospedaliero Universitaria Santa Maria della Misericordia protocol. In brief, 500 ml of whole blood from one healthy young male donor (42 years old) was collected into sterile 9 mL tubes after written

informed consent. The patient was screened and resulted negative for HIV, hepatitis B and C virus, and syphilis. The containers were left standing in an upright position to ensure clotting for at least 30 minutes at room temperature, then centrifuged at 2,000 g for 10 minutes. The supernatant serum was removed under sterile conditions in a laminar flow hood with sterile disposable syringes. The vials were frozen and left to thaw for 24 hours before treatment use.

Chemical corneal wound in rats

After intraperitoneal and topical anesthesia, 19 rats were subject to corneal damage using a chemical burn model.[2, 3, 25] In accordance to the 3R statement of the European Community (reduce, replace, refine) that encourages the use of fewer experimental animals, and to compare treatments on the same animals to reduce inter-animal variability, both eyes were used in the experiment. To induce the chemical burn, a 3-mm diameter circular filter paper soaked in NaOH was applied to the center of the cornea for 30 seconds then quickly extensively irrigated with Hank's Balanced Salt Solution (HBSS) for 60 seconds. The aim of the study was to induce corneal damage to the epithelium and anterior stroma, thus a first set of experiments was performed on 6 animals to assess corneal damage after chemical burn with 0.5 N (right eye) and 0.2 N (left eye) NaOH. Based on histological results, the protocol with 0.2 NaOH for 30 seconds was then used to generate a central corneal lesion in 13 rats for the main experiment.

Laser corneal wound in mice

After intraperitoneal and topical anesthesia, both eyes of 40 mice were subject to corneal damage by laser induced photorefractive keratectomy (PRK).[26] To induce the epithelial and stromal laser lesion, each eye was placed under an Apex Plus excimer laser (Summit,

Waltham, MA) and ablated using a 2-mm diameter central optic zone and a depth of 45-50 microns; the laser began nasally and progressed temporally.

Treatment regimen in rats

All animals were treated with topical eye drops of azythromycin 1.5% (Azyter, Laboratoires Thea, Clemrmont-Ferrand, France) for antimicrobial prophylaxis 2 times daily for 3 days after lesion.[26] Twenty-six eyes of 13 rats were divided in five treatment groups (n=5 eyes per group), which included: control, stem cells, serum, stem cells + serum, and adipose tissue. Control eyes received only antibiotic eye drops. The other 4 groups also received topical treatment applied 3 times a day for 3 consecutive days. Topical drops were administered with a delay of at least 5 minutes between applications for multiple treatment regimens. Stem cell topical eye drops were prepared daily with $1x10^5$ cells suspended in 25µL HBSS/treatment.[6] The serum group received topical application of 25 µL human serum. The stem cell+serum group were treated with both topical eye drops. Raw lipoaspirate tissue (0.1 ml) was applied to the ocular surface in the adipose group.

Treatment regimen in mice

All animals were treated with topical eye drops of azythromycin 1.5% (Azyter, Laboratoires Thea, Clemrmont-Ferrand, France) for antimicrobial prophylaxis 2 times daily for 3 days after lesion.[26] Eighty eyes of 40 rats 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 from the PGRF were intended and collected for a different ongoing study of our group, thus not included for direct comparison with this study. Control eyes received only antibiotic eye drops. The other 3 groups also received topical treatment applied 3 times a day for 5 consecutive days. Topical drops were administered with a delay of at least 5 minutes between applications for multiple treatment regimens. Stem cell topical eye

drops were prepared daily with $1x10^5$ cells suspended in 25µL HBSS/treatment.[6] The serum group received topical application of 25 µL human serum. The PGRF group received topical application of 25 µL PGRF prepared in accordance to a protocol from a previous study from one of the coauthors (JM).[13]

Ocular surface evaluation in rat eyes

Upon topical anesthesia, each treated eye was examined with a stereo biomicroscope before application of topical treatment at 20, 28, 45, 50 and 74 hours after lesion to assess corneal inflammation, opacities and other anterior surface complications (i.e. infection, perforation, etc.). Assessment time points were chosen based on laboratory and equipment availability to provide at least one data collection per day during the three days of treatment. Fluorescein sodium solution was used to evaluate the degree of the corneal epithelial defect. The defect size was determined by 2 masked graders. It was expressed in as a semi-quantitative estimate of percentage of fluorescein positive remaining area under blue light in comparison with the initial lesion area using a transparent slide with an outline of the original circular 3 mm diameter lesion. Graders gave independent masked estimates, which were adjudicated by a third grader in cases of discordance. Eyes were graded on a scale of 0 to 10 based on ranges of 10% that were rounded up to the nearest tenth, thus grade 1 showed a remaining fluorescein area that was approximately 1 to 10% of the total area; grade 2 showed an area of about 11 to 20%, etc. (Table 1 legends). The corneal surface was also examined for smoothness, clarity, and presence of neovascularization. Each animal's anterior segment was photographed with a Canon Coolpix, 5.0 megapixel camera with and without fluorescein at each clinical assessment.

Ocular surface evaluation in mice eyes

Upon topical anesthesia, each treated eye was examined with a stereo biomicroscope before application of topical treatment at 30, 54, 78, 100 and 172 hours after lesion (also referred to in this study as day 1, 2, 3, 4 and 7, respectively). This was done at each time point to assess corneal inflammation, opacities and other anterior surface complications (i.e. infection, perforation, etc.). Assessment time points were chosen based on laboratory, technician and equipment availability. Fluorescein sodium solution was used to evaluate the degree of the corneal epithelial defect. Each animal's anterior segment was photographed with a Leica EC3 digital camera equipped with a Leica S6D magnifier and 12.5x objective (Leica Microsystems, Wetzar, Germany) with and without fluorescein at each clinical assessment. The defect area was determined by the fluorescein positive remaining area under blue light (1mm=240 pixels) using Image J software (National Institutes of Health, Bethesda, MD). The corneal surface was also examined for smoothness, clarity, and presence of neovascularization.

Histological examination

Eyes were fixed in 4% paraformaldehyde and included in paraffin. The specimens were cut into 5 µm-thick tissue sections with a microtome and subjected to routine hematoxylin and eosin staining. Sections were examined under light microscope. Extent of epithelium and stromal damage was assessed for the first experimental rat eyes sacrificed at the time of corneal lesion. All other remaining rat eyes were enucleated after 74 hours. With regards to the 33 mice, 32 eyes of 16 animals were enucleated after 78 hours; while the remaining 34 eyes of 17 mice were enucleated at the end of the study at 172 hours post-lesion. Histopathological investigation included assessment of epithelium regeneration, presence of inflammation, and structural integrity of fibroblasts.[14] Representative images were collected by Leica DMD108 digital microimaging network instrument (Leica, Milan, Italy) using a 10X objective (numerical aperture 0.25).

Statistical analysis

Normality of the data distribution was assessed with the Kolmogorov-Smirnov test. Data were expressed as median ± standard deviation. Differences of the data amongst groups were analyzed with SPSS 20.0 (SPSS Inc, Chicago, ILL) for Windows program using Kruskal-Wallis and Friedman test. Multiple comparisons were performed with Dunnett's test. A P value of <0.05 was considered to be statistically significant.

7. RESULTS

ADSC were obtained from human adipose tissue aspirates following a protocol optimized by Beltrami's group for the isolation and in vitro expansion of human multipotent adult stem cells.[23] As previously shown for multipotent adults stem cells obtained from human liver, bone marrow, heart and peripheral blood, ADSC expressed the pluripotent state-specific transcription factors Oct-4, Nanog and Sox 2 (Figs. 1A-D) and were characterized by a mesenchymal stem cell immunophenotype. When assessed by flow-cytometry, ADSC highly expressed CD90, CD105, CD73, however, were mainly negative for the hematopoietic markers CD34 and CD45 (Fig. 1E). Importantly, ADSC displayed multipotency, being able to differentiate into mature cell types of all the three germ layers. Specifically, when exposed to the proper differentiation inducing conditions, ADSC were able to give rise to endodermic (Figs. 1F&G), mesodermic (Figs. 1H&I) and ectodermic derivatives (Figs. 1J&K).

As shown in the histological sections in Figures 2A&B, the normal rat cornea surface had a smooth, regular and integral epithelium. The lesion induced by 0.2 N NaOH shown in Figures 2C&D caused segmented de-epithelization, Bowman layer damage and patchy anterior stromal damage. Figures 2E&F show that the 0.5 N NaOH induced

complete ulceration and damage to the anterior and mid stroma. Based on these histological findings and the fact that animals were treated for only 3 days before being sacrificed, we used the 0.2 N NaOH model to induce a rather mild ocular surface damage.

The lesion was performed uneventfully in all rat eyes. Immediately after the chemical burn, the treated areas had a uniform, hazy ground-glass appearance. There was no sign of neovascularization or perforation in all eyes. Two animals (#10 & 11) died of hypothermia at 20 hours after anesthesia. A total of 11 animals (22 eyes) were considered in the statistical analysis. Partial re-epithelization was seen in all rats at the first time point at 20 hours. All eyes were completely epithelized by 74 hours, with the exception of 3 control eyes (#3-5). Table 1 shows all raw data regarding the percent of fluorescein positive epithelium defect at each time point for each rat eye. 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 (Table 2 & Fig. 3, $P<0.05$), with the exception of the comparisons between stem cells+serum and adipose groups at 20 hours, and between stem cells and adipose groups at 45 hours, which were not significant. No differences were found amongst stem cells, serum and stem cell+serum groups at any time point, except at 28 hours in which the epithelial defect area appeared smaller in the stem cell group compared to the others (Table 2 & Fig. 3). Eyes treated with stem cells and/or serum reached complete re-epithelization in less than 50 hours, with the exception of 1 stem cell treated eye (Figs. 3&4).

Inter-individual differences were seen between rat eyes, even amongst eyes with the same treatment (Table 1&2). To limit this variability, rats #1 to 5 were treated with stem cells on the right eye and control on the left (Table 1 & Fig. 4). The stem cell treated eyes showed faster wound healing with smaller defect areas at each time point, with differences that were significant (Table 2 & Fig. 4; $P<0.05$).

Qualitative histology assessment was performed in 11 rat eyes at 74 hours after lesion (3 control, 3 stem, 2 serum, 1 stem + serum and 2 adipose). With regards to epithelium regeneration, the control eyes showed parakeratosis with epithelium discontinuation and ongoing epithelium regeneration (Fig. 5A). Slight parakeratosis was seen in 1 stem cell and 1 serum treated eye. Whole layer epithelium regeneration was observed in all stem cell treated eyes (Fig 5B). All control eyes showed mild inflammation with marked infiltration. All other eyes showed no inflammation and little cellularity, with the exception of 1 eye treated with stem + serum that showed minimal inflammation. A moderate number of activated fibroblasts were seen in all 3 control eyes and adipose treated eyes. 1 serum treated eye and 1 stem cell treated eye showed a minimal number of activated fibroblasts. The epithelium of the stem cell eyes consisted of multiple layers and less infiltration closely resembling the native corneal epithelium (Fig. 5B).

The power of the study in calculating a difference amongst groups of 10% of the percentage of fluorescein epithelium defect, considering 5 groups and 5 eyes per group, was only 11 %. The rat experiments needed to include 45 eyes per each group to provide a power of 80%.

With regards to the mice experiments, the PRK lesion was performed uneventfully in all eyes. Immediately after the laser ablation, the treated areas had a whitish uniform, hazy appearance. There was no sign of neovascularization or perforation in all eyes. Three animals (#24, 29 & 39) died of hypothermia at 30 hours after anesthesia, and four animals (#6, 12, 38 & 28) were found dead in the cages (probably due to natural causes) before the endpoint on the 7° day. A total of 33 of 40 animals (66 eyes) were considered in the statistical analysis: 16 (32 eyes) underwent treatment for 3 days and sacrificed at 78 hours and the remaining 17 animals (34 eyes) completed the 5-day treatment regimen and then sacrificed at 172 hours.

Partial re-epithelization was seen in all rats at the first time point at 30 hours. All eyes were completely re-epithelized by 100 hours (Table 3 & Fig. 6). After the first day, the fluorescein positive corneal lesion area was significantly smaller in the stem cells groups than the control eyes (Table 3 & Fig. 7; $p<0.05$); on the second day, it was significantly larger in the controls, yet comparable between stem cell and serum treatment groups (Table 3 & Fig. 7; $p<0.02$). No differences were found amongst groups on the other days.

Inter-individual differences were seen between rat eyes, even amongst eyes with the same treatment. To limit this variability, several mice were treated with stem cells on the right eye and control on the left. The stem cell treated eyes tended to show faster wound healing with smaller defect areas at most of the earlier time points in all these eyes. Figure 8 shows examples of the fluorescein positive areas at each time point for these eyes.

8. DISCUSSION

In this study, we have shown that ADSC obtained from human processed lipoaspirate enhance corneal wound healing after chemical burn and laser ablation when compared to traditional topical therapy. The percent of epithelium fluorescein positive damage in eyes treated with stem cells (with or without serum) was smaller at each time point, which was statistically significant when compared to the control and adipose treated rat eyes (Table 2) and control mice eyes (Table 3). Stem cell treated eyes reached complete epithelium closure faster than the control and adipose treated eyes (Fig. 3 & 6). In order to limit inter-individual difference, both eyes of the same rat were compared, in which the right eye was treated with stem cells and the left eye was used as a control (Fig. 4). The data from this direct comparison showed a faster and better epithelial wound healing in the stem cell group. The preliminary data from the rat histological assessment showed that the stem cell

treated corneas had complete re-epithelization after 3 days, with less inflammatory cells and limited atypical fibroblast structure compared with the control eyes, which tended to have a more discontinuous and disorganized epithelium and stromal morphological appearance with greater infiltration (Fig. 5). The re-epithelization data and histology suggest enhanced corneal wound healing with stem cells.

Existing literature on inducing chemical corneal damage is vague and rather variable with respect to chemical agents to induce damage, concentration, method, and exposure time.[2, 3, 25, 27-29] Considering our treatment regimen consisted of only 3 days, the intent of our study was to assess treatment after an ocular surface wound of the corneal epithelium and anterior stroma without obstructing recovery and limiting complications. This was done to avoid inducing a damage that could perforate the cornea, cause neovascularization, extend to the limbus or induce severe corneal scarring. Considering the lesion induced by 0.2 N NaOH (Figs. 2C&D) caused mild damage to the epithelium and anterior stroma, while 0.5 N NaOH induced complete ulceration and mid stromal damage (Figs 2E&F), and the animals were treated for only three days before sacrificing them, we used the 0.2 N NaOH model to induce a rather mild ocular surface damage.

The designs of our experiments were directed at comparing current topical treatments used in day-to-day clinics with ADSC. The control group was treated with antibiotics for routine prophylaxis. Considering animals were treated and assessed within a period of 74 hours, azythromycin (Azyter) was chosen based on the efficacy, safety and beneficial antimicrobial prophylaxis offered by topical application 2 times daily for only 3 days.[26] Autologous serum and plasma rich in growth factors have been used in the past few years in treating ocular surface disorders such as persistent epithelial defects, severe dry eyes, chronic inflammation and autoimmune disorders.[12-14] The therapeutic benefits from these autologous blood derivatives include nutrition for the ocular surface, growth

factors, epitheliotrophic and antimicrobial support, and anti-inflammatory factors. The serum group received topical application of 25 μL human serum.

Stem cell topical eye drops were prepared daily with $1x10^5$ cells suspended in 25μL of Hank's balanced salt solution (HBSS)/treatment. The aim of the study was to apply a concentrated amount of stem cells that could be applied by topical drops. We chose this concentration of cells based on the availability of stem cells, and on the limited literature that report concentrations that range from $3x10^5$ cells suspended in HBSS,[6] $2.5x10^4$ cells suspended in 2μL HBSS,[4] and $2\ x10^5$ cells suspended in 200μL.[2] We chose to prepare the stem cell drops daily and apply them 3 times a day for 3 days similar to the other treatment regimens. The stem cells appeared to be viable and healthy under light microscope analysis after 20 hours in suspension in HBSS, however, histological and biochemical analysis was not performed to confirm this.

Our data showed that the rat and stem cell treated group had slightly better and faster re-epithelization than the serum treated group in the initial phases (Table 2, Fig. 3), even though the differences were not significant, especially considering the small number of eyes in this preliminary study. This was also the case for the mice experiments that showed statistically smaller lesions after day1 in the stem cell group, which was comparable to the serum treated eyes but less than that observed in the control group on days 2 and 3 (Table 3, Fig. 7). The serum group, stem group and combined group in the rat experiments appeared to give similar superficial wound healing response, however, this needs to be confirmed in further experiments with larger cohort that are treated and assessed over longer time periods. Moreover, in addition to re-epithelization, corneal haze and stromal opacities need to be considered in the assessment of overall corneal wound healing to determine and compare the global effect of different types of treatment. Our preliminary results do not justify treatment with a slightly more invasive stem cells technique compared to blood serum. If the true advantage of stem cell therapy lies in

enhanced stromal wound healing and less scarring and intrastromal opacities, then the slightly more invasive procedure of lipoaspiration to isolate autologous stem cells compared to simply drawing blood for serum may be justified. This issue has partially been addressed in our studies currently underway, in which histological stromal comparisons were considered in a larger group of animals that were treated for a longer time period.

It also important to note that our animal model included the use of human derived stem cells and plasma, which may have induced a variety of antigen immune responses and may have played a role in the wound healing effect in the single and combined treatment regimens. There did not seem to be an additive effect with the combined stem and serum group in the rat eyes, which may have been due a greater antigen-induced immune affect brought on by 2 the different human factors applied to the rat eye, however, the cohort is much too small to draw any valid conclusions. We are attempting to isolate autologous animal derived lipoaspirates and serum for future studies to better represent the human mode of treatment.

A point should also be made about the use of the topical anesthesia, which was used daily to assess the fluorescein stained areas and may have played a role on inducing slight ocular surface damage and delayed wound healing. The negative affects of the repeated use of these drops, however, may have influenced the cohort in a similar fashion considering that all eyes, regardless of treatment regimen, underwent the same number of ocular surface assessments with the local anesthesia.

There did not appear to be any therapeutic benefits in the adipose treated rat group, which had comparable results to the control eyes (Table 2, Fig. 3). This may partially be explained by the low concentration of ADSC in the raw lipoaspirate and because the thick lipid based tissue may have actually mechanically blocked new epithelium migration and delayed the normal epithelium closure. Moreover, the non-purified human heterogeneous

lipoaspirate may have induced a pro-inflammatory response from the bulbar and lid conjunctiva.

Our study was based on the use of ADSC for corneal wound healing because of the numerous potential benefits from utilizing this cell therapy approach. A large quantity of these autologous cells can rapidly be obtained and harvested from an easily accessible reservoir, which requires mini-invasive procedures under local anesthesia. The frequency of these multipotent cells normally found in numerous tissues throughout the body is much higher in adipose tissue; more than a 100 fold higher amount of cells in adipose tissue when compared to the amount of MSC obtained from bone marrow.[19] Higher numbers of primary cells may entail the need of less passages for isolation and harvesting, thus limiting the risk of culture-induced chromosomal abnormalities and infection. Moreover, numerous studies and phase I and II clinical trials[22] in the past decade have reported the promising clinical utility of ADSC in a wide variety of tissue regeneration models, ranging form plastic surgery fillers,[30] muscle,[31] bone[32] and heart.[23, 33]

The physiological roles of ADSC are diverse and promising in tissue regeneration and wound healing. Numerous studies have focused on the use of MSC from bone marrow to treat various pathologies and disorders in the past 40 years, however, it was the work by Zuk et al in 2002 that showed a large reservoir of functional MSC in adipose tissue.[17] ADSC isolated from the stroma-vascular fraction of adipose tissue obtained from lipoaspirate have been shown to meet the minimal set of 4 criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular therapy[34] used to define functional human MSC, which include: plastic-adherency in standard culture conditions;[17, 18, 24] ability for osteogenic, adipogenic and chondrogenic differentiation;[17, 18, 24] the expression of specific CD markers (i.e. CD10, CD 13, CD 29, CD44, etc.),[17, 23, 24, 35] and the lack of certain hematopoietic and leukocyte antigen markers (i.e. CD45, CD14, CD16, etc).[17, 35] Several molecular and biochemical characterization

studies have shown that ADSC derived from lipoaspirates are capable of multiple mesodermal lineage differentiation, considering that several lineage specific genes and proteins for bone, fat, cartilage, muscle and neural-like tissue can be induced in vitro with selective medium cultures.[17, 18] In addition to their extensive proliferation potential and multilineage differentiation, ADSC can interact and affect the immune system response to injury, by the down-regulation of proinflammatory factors and production of several trophic factors.[2, 16, 36] Several reports have shown the immunoregulatory properties of MSC, which include the inhibition of T-cells,[37] increase in tumor necrosis factor (TNF) from dentritic cells,[38] increase in regulatory T-cells,[38] block of antigen producing cell maturation,[39] increase in immunosuppressive cytokine interleukin (IL)-10 and TNF-β and decrease in IL-2.[2] The endocrine function of adipose tissue is evident through the secretion of numerous growth factors (GF) like epidermal GF, vascular endothelial GF, basic fibroblast GF, keratinocyte GF and platelet derived GF.[35]

There are limited studies in current literature regarding the use of ADSC in ocular surface and stromal wound healing. Arnalich-Montiel et al were one of the first groups to publish a study using human ADSC to regenerate the cornea stroma in a rabbit model a few years ago.[6] The experiments involved inserting ADSC obtained from human lipoaspirate in an intrastromal pocket created with a femtosecond laser. The first set of experiments showed that the harvested stem cells exhibited multipotency, in that they could be harvested into chondrogenic and osteogenic lineages. Clinical and histological assessment proved that the transplanted cells were safe, caused no immune reaction,[40] maintained corneal transparency and did not show any clinical differences with the control eye. The transplanted multipotent ADSC acquired a dendritic morphology similar to keratocytes and appeared to behave like regular rabbit corneal stroma cells in situ, being able to produce collagen I &VI but not collagen III & IV, which are typical characteristics of keratocytes.[41] Polymerase chain reaction (PCR) experiments showed that these

transplanted cells exhibited human keratocan and ALDH expression, which are typical in corneal cells, and were not found in the control group. The authors justly concluded that ADSC could be a viable cell source for stromal repopulation and repair in corneal pathologies.

In vitro reports have provided immunohistochemical and molecular evidence that ADSC cultured in specific keratocyte-differentiation conditions can adopt a keratocyte phenotype, which can synthesize and secrete cornea specific keratocan mRNA and keratocyte specific proteins.[5] Du et al showed that isolated human corneal stem cells injected in murine corneas restored stromal defects and maintained corneal transparency.[4] The ability of ADSC to remodel tissue in a manner consistent with normal organogenesis could explain the reduced amount of scarring and residual opacities during the wound healing process. Rat immunofluorescent studies involving chemically damaged corneas treated with human bone marrow MSC plated on amniotic membrane showed the therapeutic effect of treatment may have been associated with the inhibition of inflammation and inflammation-related angiogenesis after transplantation.[3] MSC obtained from rat primary cell lines also exhibited anti-inflammatory and anti-angiogenic activity in chemically burned rat corneas.[2] The study showed that both the MSC and culture medium derived from MSC reduced corneal inflammation and neovascularization, decreased IL-2 and IFN, increased IL-10 and IL-6, reduced infiltration of CD4+ cells and upregulated the expression of thrombospondin-1, which the authors attributed to probable paracrine mechanisms, cell-to-cell contact, and the continuous secretion of soluble factors and cytokines from MSC and activated cells in situ.[2]

The presence of corneal limbal stem cells (LSC) was first discovered in the late 1980's.[42] These slow-cycling subpopulation of epithelial basal cells located in the peripheral limbus of the cornea were found to have a substantial proliferating capacity. Autologous and allogenic LSC transplants are surgical option,[43] however, donor tissues

are limited and autologous transplants may give rise to iatrogenic damage to the healthy fellow eye or at times the fellow eye is not healthy. Tissue engineering pertains to LSC as a cell source for epithelial reconstruction, thus may have limiting therapeutic effects on damaged stroma. Recent studies have reported the presence of MSC in the human limbal biopsies having similar immunophenotype and immunocytochemical markers to those present in bone marrow derived MSC.[44] Choong et al. showed that isolated corneal keratocytes having fibroblastoid morphology expressing similar CD antigens to bone marrow MSC could be isolated from finely chopped human cornea tissues.

With regards to the clinical use of ADSC for ocular surface wounds in humans, a case report has recently been published by a group in Greece.[4545] A young male patient with stable keratoconus showed post-traumatic corneal epithelial defect in his right eye. Several months of traditional therapy was not effective in treating the persistent sterile corneal ulcer. He was scheduled for penetrating keratoplasty due to risk of corneal perforation and severe stromal opacity that reduced central visual acuity, which was 20/100. In the meantime, the patient agreed to undergo an experimental treatment involving topical application of autologous ADSC, which was obtained by lipoaspiration of subcutaneous adipose tissue from the lumbar area. The ADSC were isolated from the lipoaspirate and applied to the bottom of the ulcer, followed by closure of the lid with a pressure eye patch for 24 hours. Corneal healing was observed after 11 days. At six months after treatment, the patient still did not require surgery, visual acuity was improved to 20/40, central corneal thickness was 476 μm, and corneal transparency improved with mild residual anterior stromal opacification.

We included the extremely low power of our rat study in the results section to indicate that a rather large group of animals are needed to give results that are significant. Although the mice experiments were based on a larger group of animals, additional eyes needed to be included to reveal slight statistical differences (if any) pertaining to epithelium

repair throughout time, especially between stem cell and serum treated eyes. Moreover, future studies assessing corneal stromal haze and histological repair over longer time periods (several weeks and months) are surely needed to determine if the main advantages of the slightly more invasive autologous adipose stem cell approach outweigh the benefits of serum or traditional antibiotic treatments. Our study confirmed the biosafety, immunogenicity and efficacy of stem cells, which proved to be comparable to the serum treatment and surely better than the control eyes treated solely with traditional topical antibiotics. The aim of this preliminary study was to assess the probable therapeutic affects of ADSC without having to sacrifice a large group of animals, especially considering the extremely limiting information currently available in literature. The data presented in this study are not intended to assess statistically significant results, however, to provide helpful insights to guide future studies in this field.

In conclusion, our preliminary study is limited by the small number of animals considered, the brief period time for treatment and clinical assessment, semi-quantitative data and the non-extensive histological evaluation. 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. Epithelial recovery, inflammation, corneal haze, and quantitative histological assessments are currently being compared between the different treatment arms. The aim in our future studies are to determine whether or not the mid to long term healing processes that determine stromal haze and loss of corneal transparency can be positively influenced by the presence of topically applied ADSC. These cells are multipotent and have the potential to differentiate towards a keratocyte stromal lineage, thus theoretically appear to be a promising therapeutic alternative and advantageous compared to treatments currently utilized in clinical practice. Moreover, the concentration

of stem cells, viability of cells in isolated medium, number of applications per day and duration of treatment still need to be determined if this novel therapy proves to be beneficial.

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 mechanisms behind ADSC may range from ADSC transdifferentiation and substitution of non-functional or abnormal keratocytes, limbal stem cell repopulation, paracrine mechanisms, cell-to-cell contact and signaling, stimulation of residing MSC, production of soluble cytokines and factors that promote normal tissue organogenesis. Future studies are definitely needed to better understand the potential therapeutic benefits offered by ADSC that are already being used in a variety of clinical studies involving different organs and human pathologies. The clinical use of ADSC looks promising, especially considering the abundant and easily accessible reservoir of autologous tissue, relatively low health costs involved, and the use of cells that show multipotency, and anti-inflammatory and trophic characteristics, which may promote proper corneal epithelium and stromal wound healing.

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10. TABLES

Table 1. Estimated percent of fluorescein positive epithelium defect (10% intervals) at each time point for each rat treatment group

group	rat #/eye	20h	28h	45h	50h	74h
control	1L	7	4	3	1	0
control	2L	7	5	3	1	0
control	3L	6	5	4	2	1
control	4L	5	4	4	2	1
control	5L	7	4	3	1	1
stem	1R	5	1	0	0	0
stem	2R	6	2	1	0	0
stem	3R	4	2	1	1	0
stem	4R	2	1	2	0	0
stem	5R	4	2	1	0	0
serum	6R	5	3	3	0	0
serum	7R	4	3	1	0	0
serum	8R	6	4	1	0	0
serum	9R	2	2	1	0	0
stem+serum	6L	6	1	1	0	0
stem+serum	7L	5	4	0	0	0
stem+serum	8L	4	1	0	0	0
stem+serum	12L	5	5	1	0	0
adipose	9L	4	3	1	1	0
adipose	12R	7	7	1	1	0
adipose	13L	6	6	1	1	0

Legends for fluorescein groups

Grading scale	% fluor. positive interval
0	0
1	1-10
2	11-20
3	21-30
4	31-40
5	41-50
6	51-60
7	61-70
8	71-80
9	81-90
10	91-100

Table 2. Percent of fluorescein positive epithelium defect (10% intervals) for each rat group at each time point						
time	**control (median±SD)**	**stem cells (median±SD)**	**serum (median±SD)**	**stem+serum (median±SD)**	**adipose (median±SD)**	**p value***
20 h	7 ± 0.89	4 ± 1.5	4.5 ± 1.71	5 ± 0.82	6 ± 1.52	0.097
28 h	4 ± 0.56	2 ± 0.55 ^^	3 ± 0.82	2.5 ± 2.06	6 ± 2.08	0.025
45 h	3 ± 0.55^^^	1 ± 0.7	0 ± 1.0	0.5± 0.58	1 ± 0	0.008
50 h	1 ± 0.55	0 ± 0.45^^	0 ± 0^^	0 ± 0^^	1 ± 0	0.002
74 h	1 ± 0.55^^^	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.031
p value§	0.0001	0.0001	0.0001	0.0001	0.0001	

SD= standard deviation; * = Kruskal-Wallis test; § = Friedman test; ^ = significantly higher in comparison with stem cell and serum groups; ^^ = significantly lower in comparison with control and adipose groups; ^^^ = significantly higher in comparison with the other groups; legends for fluorescein intervals listed in Table 1.

Table 3. Median fluorescein positive corneal lesion area (mm^2) in mice for each group over time

Time after lesion Day (hours)	controls (median±SE)	basic serum (median±SE)	stem cells (median±SE)	p value*
Day 1 (30h)	1.25 ± 0.80[a]	0.90 ± 0.68	0.83 ± 0.42	0.048
Day 2 (54h)	0.26 ± 0.39[b]	0.07 ± 0.40	0.08 ± 0.20	0.018
Day 3 (78h)	0.05 ± 0.29	0.04 ± 0.32	0.01 ± 0.14	0.127
Day 4 (100h)	0.00 ± 0.05	0.00 ± 0.00	0.00 ± 0.03	0.202
Day 7 (172h)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000
p value^	0.0001	0.0001	0.0001	

* = Kruskal-Wallis test; ^ = Friedman test; [a] = significantly higher than stem cells group; [b] = significantly higher than the other groups

11. FIGURES

Figure 1

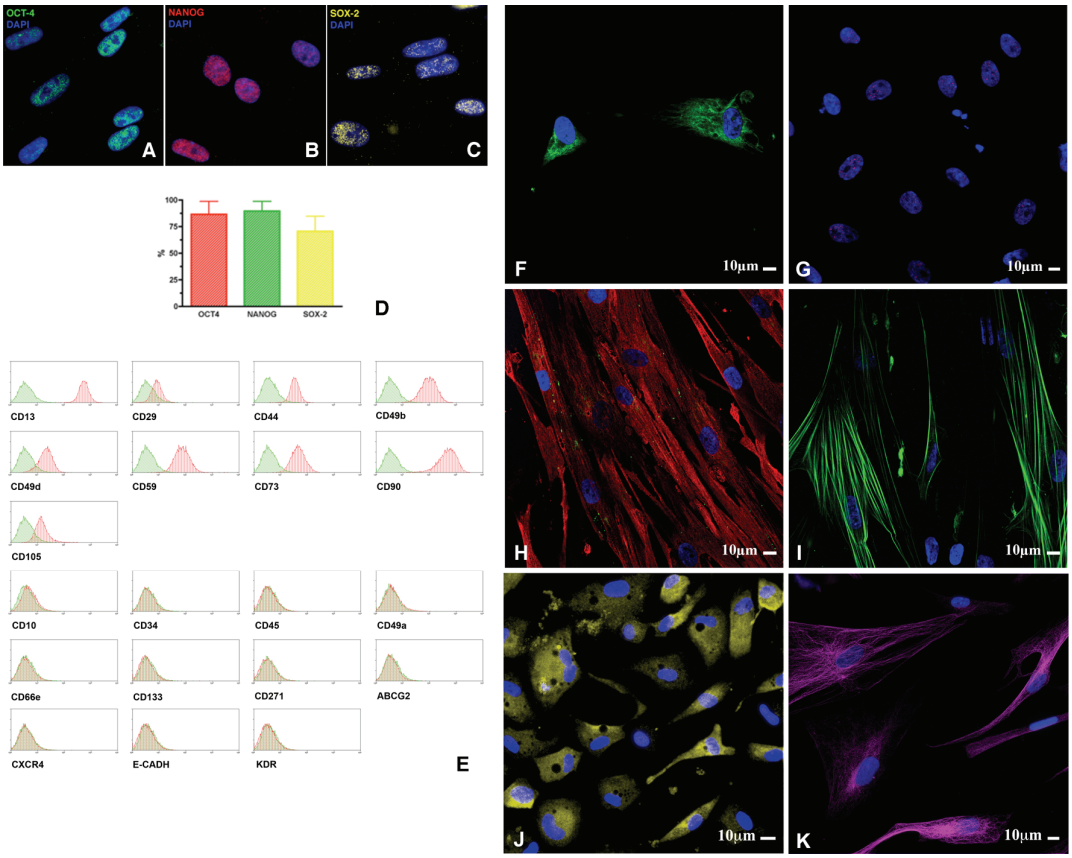


Figure 1: ADSC expressed Oct-4 (green fluorescence; **A**), Nanog (red fluorescence; **B**) and Sox-2 (yellow fluorescence; **C**). Quantification of pluripotent state-specific transcription factor expression (**D**). Data are presented as mean±standard deviation. Representative flow cytometry histograms of ADSC (**E**). Plots show isotype control IgG-staining profile (green histogram) versus specific antibody staining (red histogram). Multipotency of ADSC (**F-K**). Endodermic differentiation (**F&G**). Cells cultured for in a medium added with HGF and FGF-4 became positive for cytokeratins 8-18-19 (green fluorescence; **F**) and for GATA-4 (green dots; **G**). Myocyte differentiation (**H&I**). A low fraction of ADSC in differentiation medium express the myocyte-specific filament SMA (green fluorescence; **I**), while the large part of the cells express a-sarcomeric actin in the cytoplasm (red fluorescence; **H**) and connexin-43 on the cell surface (green fluorescence; **H**). Neuroectodermic differentiation (**J&K)**. ADSC in neurogenic medium acquired the expression of neuron specific enolase (yellow fluorescence; **J**) and tubulin beta-3 (magenta fluorescence; **K**). Nuclei are depicted by the blue fluorescence of DAPI staining.

Figure 2

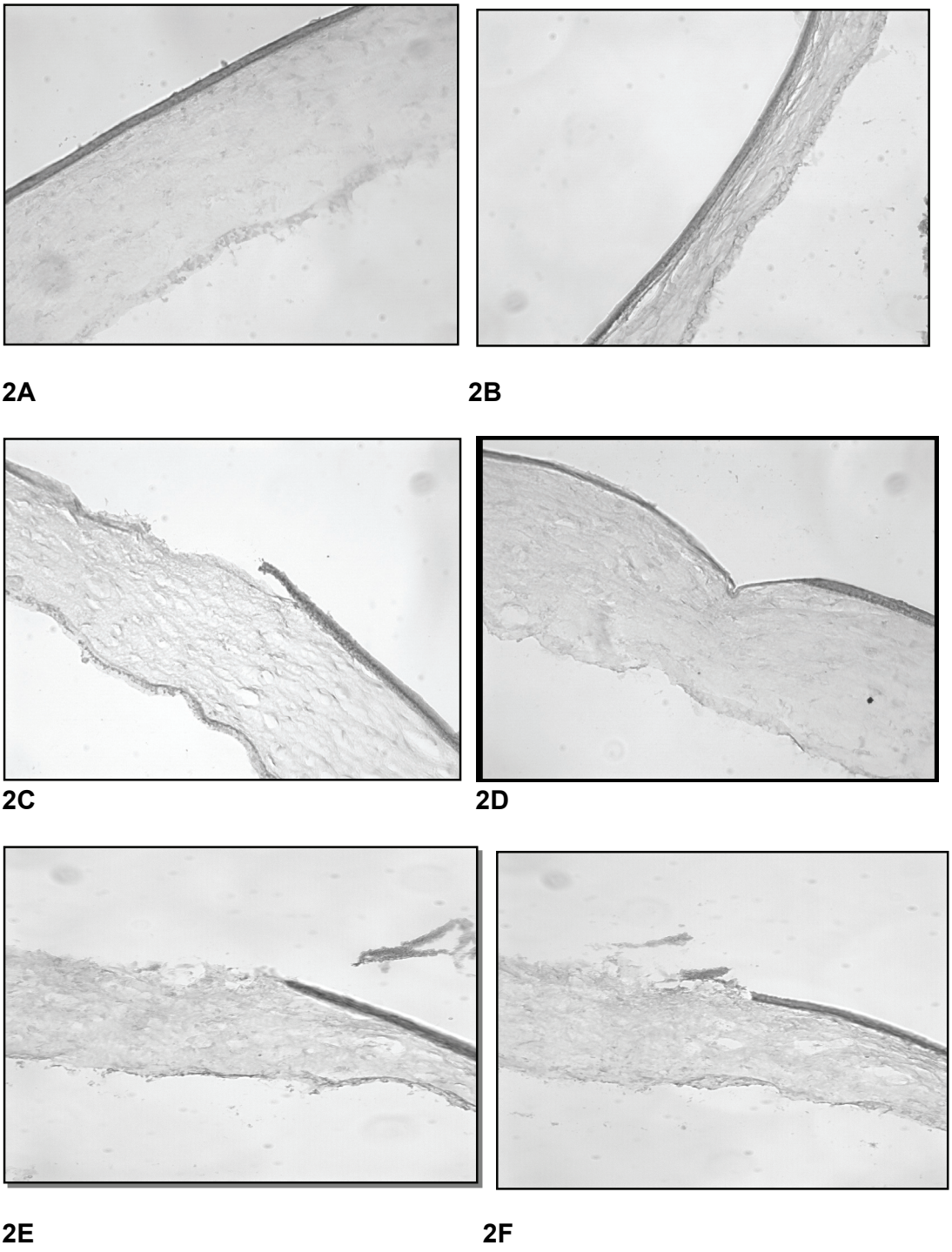


Figure 2: Light microscope findings on normal and chemically wounded rat corneas. The epithelium layer appears as a dark band positioned on the top side of each figure. Normal rat cornea surface with no lesion had a smooth, regular and integral epithelium (**A&B**). The chemical burn with 0.2 N NaOH for 30 seconds caused de-epithelization, Bowman layer damage and patchy anterior stromal damage (**C&D**). Chemical burn with 0.5 L NaOH for 30 seconds induced complete ulceration and damage to the anterior and mid stroma (**E&F**).

Figure 3

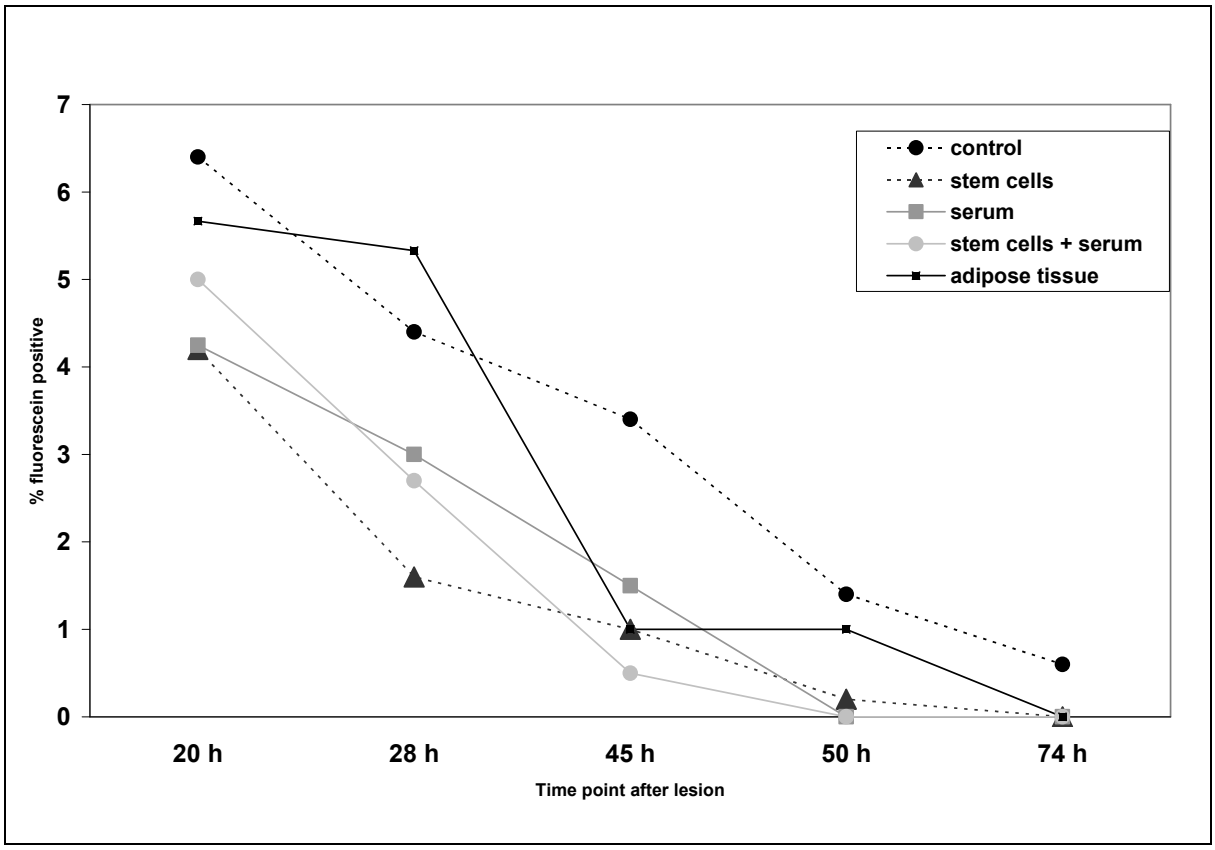


Figure 3: Median epithelium defect area (grouped in 10% intervals) over time expressed as a percent of fluorescein positive area remaining at each time point for each treatment group in the rat eyes.

Figure 4

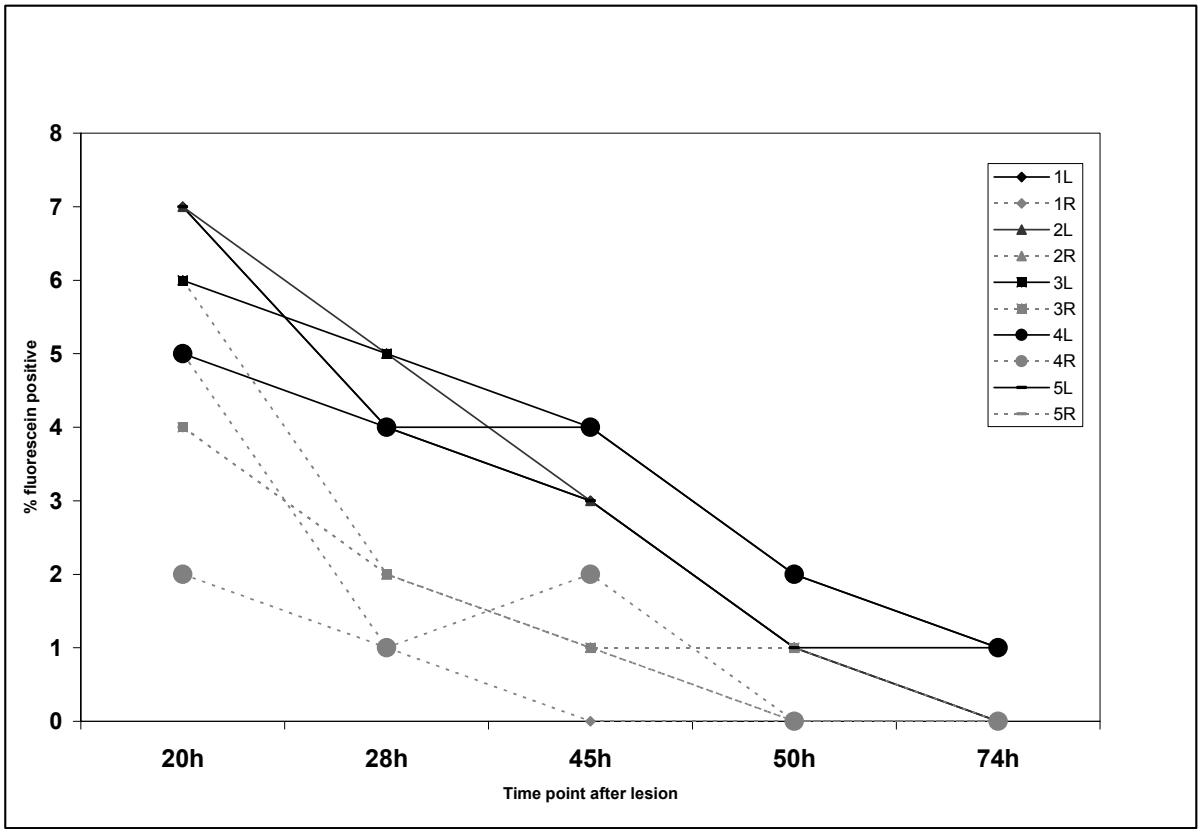
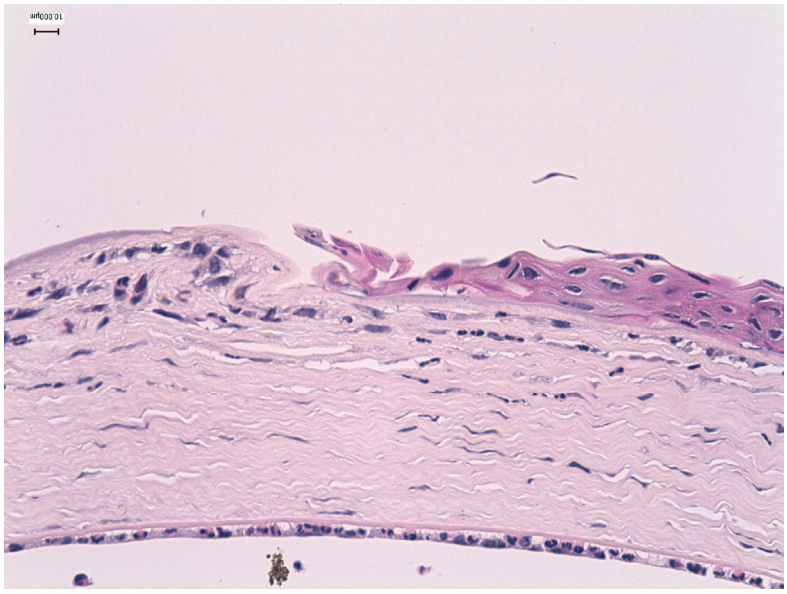
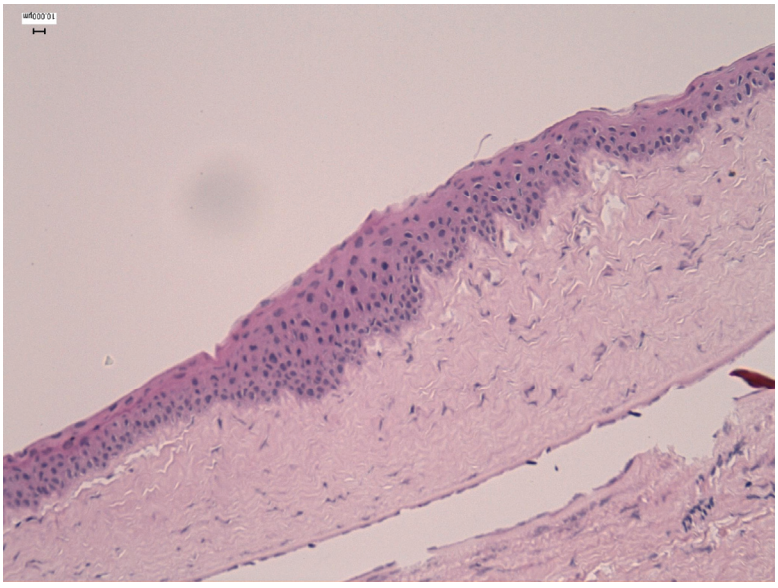


Figure 4: Percent of epithelium defect area (grouped in 10% intervals) over time for control (left eyes shown in black solid lines) and stem cell treated (right eye shown in grey dotted lines) eyes for rats #1-5.

Figure 5



5A



5B

Figure 5: Light microscope findings of corneal epithelium wound healing after 74 hours for control eye (**A**) and stem cell treated eyes (**B**) stained with hematoxylin and eosin (x100). The epithelium is shown as the darker stained tissue on the top side of each figure. Stem cell treated corneas showed complete re-epithelization, with less inflammatory cells and limited atypical fibroblast structure compared with the control eyes.

Figure 6

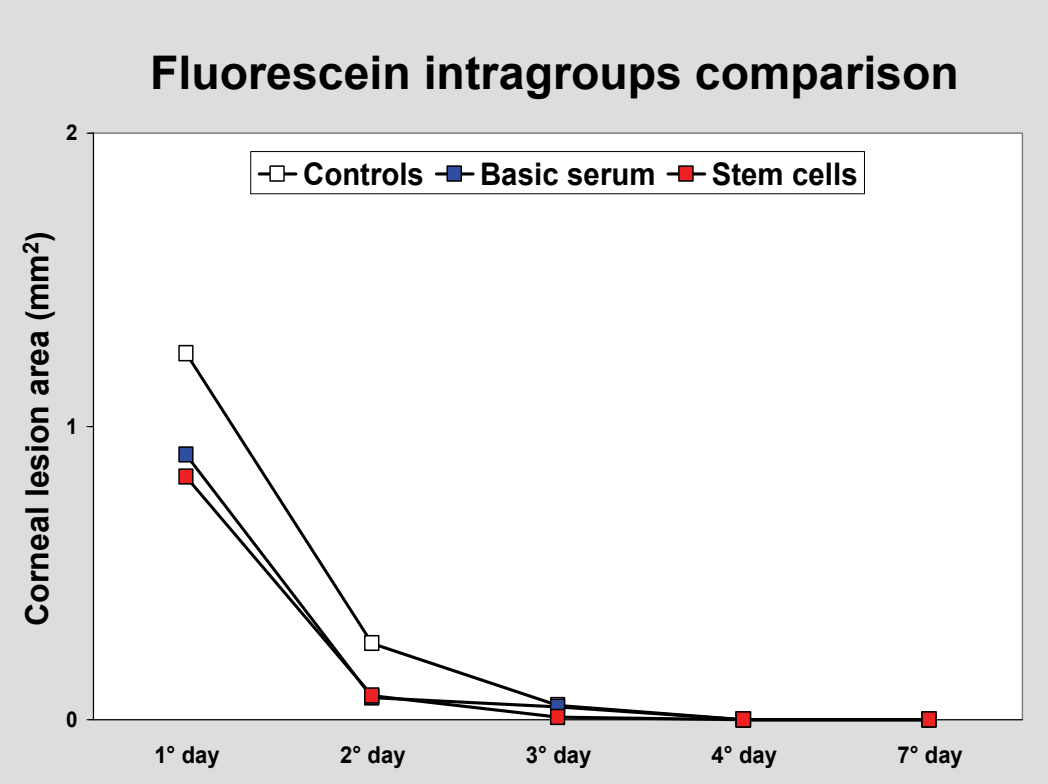


Figure 6: Intergroup comparisons of median epithelium defect area over time for each treatment group in the mice eyes.

Figure 7

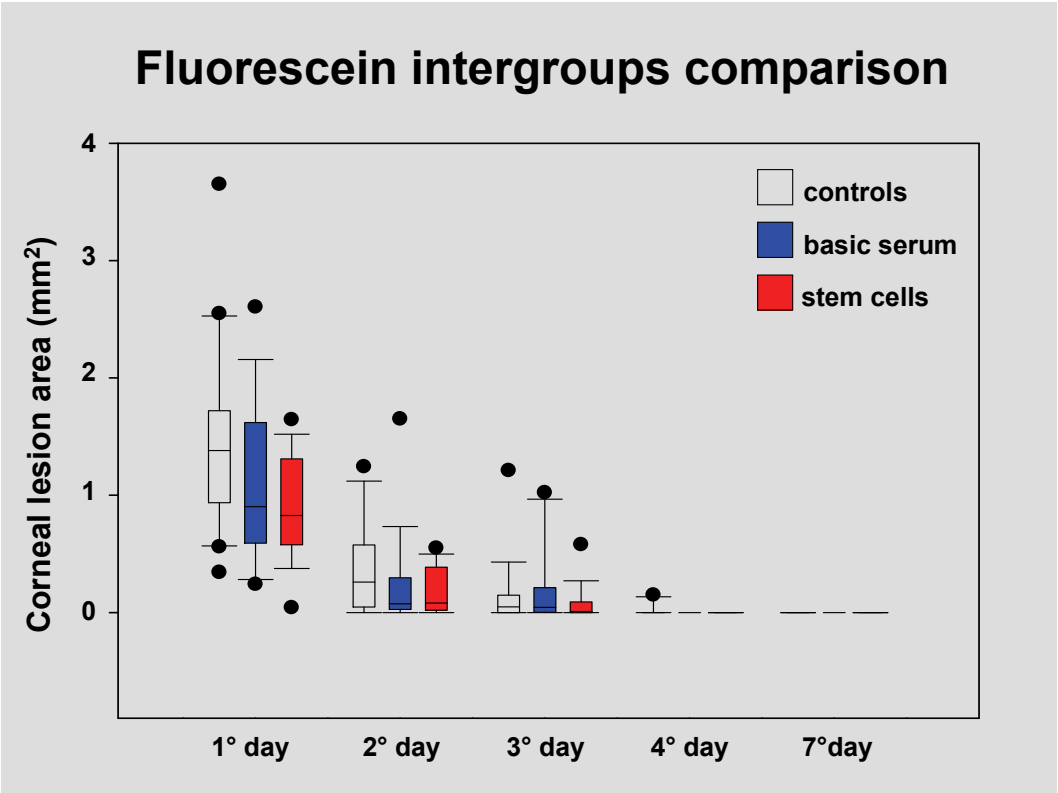


Figure 7: Box plots of median epithelium defect area over time at each time point for each treatment group in the mice eyes.

Figure 8

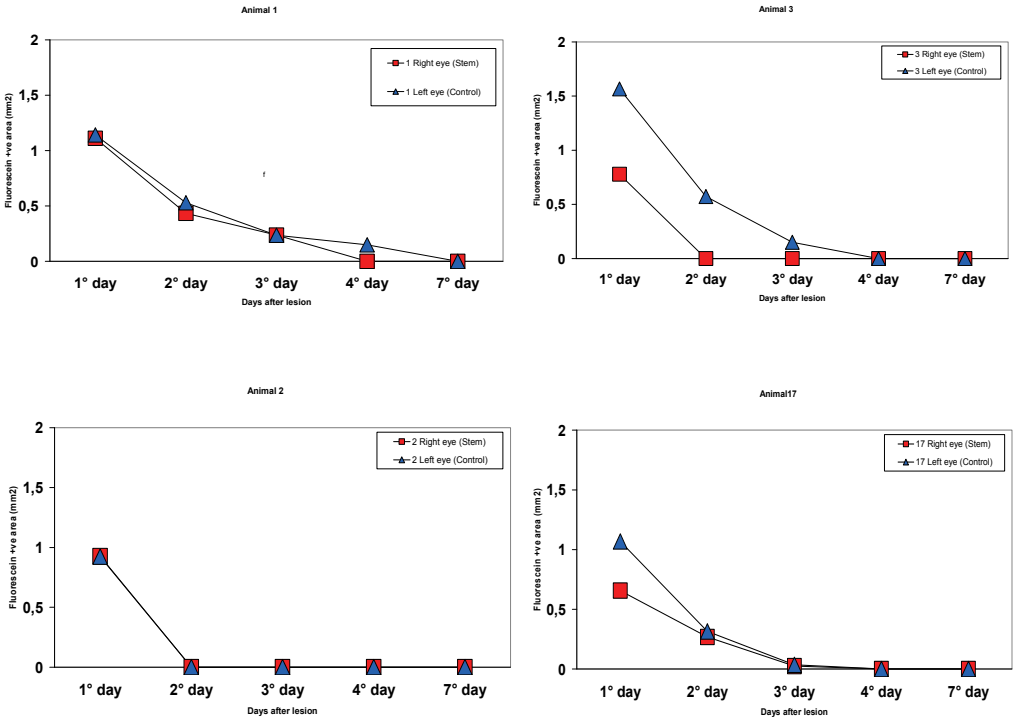


Figure 8: Fluorescein positive areas at each time for animals # 1, 2, 3 & 17. For intraindividual comparisons, the right eye (red squares) of each animal was treated with stem cells and the left eye (blue triangles) was treated with the control regimen. The stem cell eyes tended to show better epithelial wound healing than the control eyes in the first 3 days with smaller fluorescein areas and faster total re-epithelization.

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