Histological and Immunohistochemichal Study of the Possible Protective Effect of Antioxidants on Albino Rat Cornea After Ethanol Exposure

Thesis

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ABSTRACT

The human eye is subjected to ethanol during Laser Subepithelial Keratomileusis surgery (LASEK) in order to create an epithelial flap. It is known that ethanol induces corneal damage attributed to the action of reactive oxygen species.

The aim of this study was to evaluate the possible protective role of vitamin E and vitamin C on corneal damage, apoptosis and inflammation induced by ethanol application.

Twenty eight adult male albino rats (150-200 g) were divided into 4 groups, 7 rats each. Group I served as control. In group II corneas were exposed to 20% ethanol (2 drops) for 30 seconds. In group III vitamin E (100mg/1ml) was applied topically on corneas exposed to ethanol. In group IV vitamin C (100mg/1ml) was applied topically on corneas exposed to ethanol. Animals were sacrificed after 24 hours. The eyeballs were removed and processed. Sections were stained with H&E, Masson trichrome and immunohistochemical stain for FADD (to detect apoptosis) and for COX-2 (to detect inflammation). Morphometric analysis was done for the apoptotic index of epithelial nuclei, number of stromal keratocytes and blood vessels as well as area % of COX-2 immunoexpression.

In group I, few apoptotic nuclei were seen. In group II corneas were markedly affected, an epithelial flap was formed, one basal cell layer with dark nuclei, neovascularization and multinucleated giant cells were seen in a distorted stroma. Apoptotic index values and COX-2 expression were increased when compared to the other groups. In groups III & IV corneas appeared not completely recovered.

Vitamin E or vitamin C application appeared to be effective in the protection of corneal damage induced by ethanol application.

KEY WORDS: *Albino rat, Cornea, Ethanol, LASEK, Apoptosis, FADD, Fas, COX-2, Neovascularization, Vitamin E, Vitamin C.*

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Dalia Ibrahim

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LIST OF ABBREVIATIONS

LASEK	Laser subepithelial keratomileusis
LASIK	Laser in situ keratomileusis
PRK	Photorefractive keratectomy
RNA	Ribonucleic acid
ATPase	Adenosine triphosphatase
ATP	Adenosine triphosphate
FasL	Fas ligand
FADD	Fas-associated death domain protein
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
ROS	Reactive oxygen species
PGE2	Prostaglandin E2
mRNA	Messenger ribonucleic acid
TUNEL	TdT-mediated dUTP nick-end labeling
PMN	Polymorphnuclear leucocyte
H&E	Hematoxylin and Eosin
VEGF	Vascular endothelial growth factor
IL	Interleukin
HLA	Human lymphocyte antigen

INTRODUCTION AND AIM OF THE WORK

The cornea is highly specialized to refract and transmit light. It is covered by a non-keratinized stratified squamous epithelium. This epithelium is responsible for maintaining ocular surface integrity, which is essential for vision and also provides an initial barrier to tears and to the intraocular environment ***(Nishida, 2005).***

Laser subepithelial keratomileusis (LASEK) is a relatively new refractive surgical technique that is applied to avoid the disadvantages of laser in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK). Theoretically, LASEK produces fast epithelial healing and visual recovery, with less stromal haze and postoperative pain than PRK. It also offers the advantages of avoiding the flap- and interface-related complications of LASIK as well as avoiding the difficulty in predicting flap thickness and ablation depth ***(Song and Joo, 2004).***

In LASEK, 20% ethanol is applied for 30 seconds to displace the corneal epithelium prior to laser ablation. After that, the epithelium is repositioned over the ablated surface. The epithelial flap is expected to remain viable and limit corneal wound healing. However, epithelial damage is common after LASEK, and causes delayed visual recovery and pain in early postoperative period ***(Bilgihan et al., 2005).***

These complications occur because ethanol is cytotoxic, as it induces apoptosis in a variety of non-ocular tissues and recently it has been found to induce apoptosis in corneal epithelium. So, a major concern with the use of ethanol in the corneas is about the continued viability of the epithelium after the procedure as it is related to wound healing and visual rehabilitation ***(Sosne et al., 2004).***

Moreover, ***in 2006 Miyamoto et al.*** found that ethanol may activate an inflammatory cascade in the cornea resulting in corneal haze after LASEK. This suggests the use of anti-inflammatory treatment before this operation although it may impair corneal wound healing.

Bilgihan et al. (2005) suggested that the use of antioxidants might avoid the corneal epithelial apoptosis induced by ethanol, so it may have a role in limiting wound healing after LASEK.

The aim of the work:

This study aimed at evaluating the harmful effects of ethanol (20%) when applied to the corneas of albino rats for 30 seconds simulating what is happening in LASEK technique. In addition, the study aimed at testing the possible protective effects of antioxidants (vitamin E and vitamin C) on the corneas of the rats after ethanol application.

NORMAL CORNEA

Microscopic structure:

The cornea is a transparent avascular tissue that is exposed to the external environment. The anterior corneal surface is covered by the tear film, and the posterior surface is bathed directly by the aqueous humor. The transparent cornea is continuous with the opaque sclera and the semi-transparent conjunctiva. The highly vascularized limbus, which contains a reservoir of pluripotential stem cells, constitutes the transition zone between the cornea and the sclera ***(Nishida, 2005).***

The cell types that constitute the cornea are epithelial cells, keratocytes, and endothelial cells. Epithelial cells are derived from the epidermal ectoderm, whereas keratocytes and endothelial cells are of neural crest (neuroectodermal) origin. The precise arrangement of the various components of the cornea contributes to its transparency and strength. The cornea consists of three different cellular layers and two interfaces: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium. Components of the cornea interact with each other to maintain the integrity and the function of the tissue ***(Maurice, 1984).***

Epithelium:

The corneal and conjunctival epithelia are continuous and together form the ocular surface. Although their characteristics differ, they cooperate to provide the biodefense system of the anterior surface of the eye. The corneal epithelium is composed of non-keratinized, stratified squamous epithelial cells. The thickness of the corneal epithelium is approximately 50 µm, which is about 10% of the total thickness of the cornea, and is constant over the entire corneal surface ***(Sack et al., 2001).***

The structure of the corneal epithelium is similar to that of the epidermis of the skin. An important difference between the two tissues is that the corneal epithelium is not keratinized, although it may become so under pathological conditions such as vitamin A deficiency ***(Ubels and MacRae, 1984).***

The corneal epithelium consists of five or six layers of three different types of epithelial cells: two or three layers of superficial flat cells, two or three layers of wing cells, and a monolayer of columnar basal cells, which adhere to the basement membrane adjacent to Bowman’s layer. Only the basal cells of the corneal epithelium proliferate, and the daughter cells differentiate into wing cells and subsequently into superficial cells, gradually emerging at the corneal surface. The differentiation process requires about 7-14 days, after which the superficial cells are desquamated into the tear film ***(Hanna et al., 1961).*** Ultraviolet radiation, hypoxia, chemicals and mechanical stress induce apoptosis and desquamation of the corneal epithelial cells. It remains unclear whether apoptosis contributes to cell turnover or not in the corneal epithelium under normal conditions ***(Estil et al., 2000).***

The epithelium, together with the tear film, contributes to maintenance of the optically smooth corneal surface. In individuals with dry eye, the surface of the corneal epithelium is dehydrated and becomes pitted and irregular. This loss of smoothness of the epithelial surface results in degradation of the optical images and consequent blurred vision ***(Pfister, 1973).***

Another important physiological role of the corneal epithelium is to provide a barrier against external biological and chemical insults as it is subjected to injury or invasion by microbes. The presence of junctional complexes between adjacent corneal epithelial cells prevents the passage of chemical substances into the deeper layers of the cornea. Defects of the corneal epithelium allow the penetration of the tear fluid into the stroma, resulting in stromal edema that interferes with the transmission of light through the cornea. Rapid renewal and the formation of inter-cellular junctions between the corneal epithelial cells also help to protect the cornea from microbial attacks ***(Yokoi and Kinoshita, 1995).***

Suzuki et al. (2000) found that both cell-cell and cell-matrix interactions are important for maintenance of normal stratified structure and physiological functions of the corneal epithelium. Tight junctions, together with the interdigitations of cell membranes, provide a highly effective barrier preventing the penetration of the tear fluid and its components. Adherent junctions and desmosomes are present in all layers of the corneal epithelium, whereas gap junctions are present between the wing cells and the basal cells allowing the passage of small molecules.

Components of the intracellular cytoskeleton (actin filaments, microtubules, and intermediate filaments) contribute to the shape and motility of cells. Cellular components of the epithelium also play an important role in the immunology. Dendritic Langerhans cells (specialized macrophages derived from bone marrow and implicated in antigen processing) are abundant at the periphery of corneal epithelium (near the corneal limbus) but are not present in the central region of the normal cornea ***(Gillette et al., 1982).***

The Langerhans cells of the corneal epithelium express human lymphocyte antigen (HLA) class II molecules and present antigens to T lymphocytes. Their numbers are increased in conditions characterized by ocular inflammation and are reduced after treatment with corticosteroids. Injury to the central cornea results in rapid migration of peripheral Langerhans cells to the damaged area ***(Whitsett and Stulting, 1984).***

I) Superficial cells:

The surface of the corneal epithelium contains two to three layers of terminally differentiated superficial cells. These cells are flat polygonal with a diameter of 40-60 µm and a thickness of 2-6 µm. Their surface is covered with microvilli that greatly increase the surface area of each cell and thereby promote the active uptake of oxygen and nutrients from tear fluid. With the aid of scanning electron microscope, two different types of superficial cells were described in the corneal epithelium:

- Large dark mature cells that have dense coat of microvilli and are about to desquamate into the tear film.
- Small light younger cells with fewer microvilli.

The superficial cells are well-differentiated. They do not proliferate, exhibit a low metabolic activity, and contain fewer organelles: some free ribosomes as well as fragments of endoplasmic reticulum, few mitochondria and a poorly developed Golgi complex. They also have less RNA than do the other types of corneal epithelial cells ***(Pfister, 1973).***

Numerous glycoprotein and glycolipid molecules are embedded in the hydrophobic lipid bilayer that constitutes the cell membrane of the epithelial cells. These oligosaccharide-containing molecules form floating particles in the membrane. They are called glycocalyx which present hydrophilic properties on the anterior surface of the superficial epithelial cells. This glycocalyx interacts with the mucinous layer of the tear film and helps to maintain the trilayered structure of the latter. Loss of the glycocalyx, by frequent application of eye preservatives, leads to instability of the tear film that results in dry eye ***(Argueso and Gipson 2001).***

The superficial cells of the cornea are joined together by desmosomes and tight junctions, which prevent the passage of substances through the inter-cellular spaces. Interruption of the continuity of corneal epithelium allows aqueous material to penetrate into the corneal stroma ***(Yokoi and Kinoshita, 1995).***

II) Wing cells:

There are two to three layers of wing cells beneath the superficial cells. They are called so because of the characteristic wing like shape. Wing cells are in intermediate state of differentiation between basal and superficial cells and are rich in intracellular tonofilaments called keratin. The cell membranes of adjacent wing cells interdigitate. Numerous desmosomes and gap junctions are present between wing cells. Cytoplasmic organelles are relatively sparse in these cells ***(Doran et al., 1980).***

III) Basal cells:

A single layer of cuboidal basal cells is seen resting on the basement membrane. They are the only cells of corneal epithelium that possess mitotic activity. Basal cells are the source of wing cells and superficial cells. Consistent with their mitotic activity, they contain more intracellular organelles, free ribosomes, rough endoplasmic reticulum, mitochondria, centrioles, microfilaments, microtubules and glycogen granules than do wing cells or superficial cells. The nucleus of each basal cell is displaced interiorly ***(Beebe and Masters, 1996).***

Neighboring basal cells interdigitate laterally and are joined by desmosomes, gap junctions and adherent junctions. The posterior surface of basal cells is flat and adjoins the basement membrane via hemidesmosomes that are linked to anchoring fibrils of type VII collagen. The anchoring fibrils penetrate into the stroma where they form anchoring plaques together with type I collagen, a major component of the stroma ***(Gipson et al., 1987).***

IV) Basement membrane:

As in epithelia in other parts of the body, basal cells of the corneal epithelium secrete the components necessary for formation of the basement membrane. It is 40-60 nm thick and is composed of a pale layer (lamina lucida) immediately posterior to the cell membrane of the basal cells and an electron dense layer (lamina densa). The major components of the basement membrane are type IV collagen and laminin, both are synthesized and secreted by basal corneal epithelial cells. The basement membrane also contains heparan sulfate and fibrin ***(Gipson, 1989).***

The presence of the basement membrane between the basal epithelium and the stroma fixes the epithelial cells and provides a matrix on which cells can migrate. Also, it is thought to be important for maintenance of the stratified well organized corneal epithelium. Thus, it plays a prominent role in epithelial wound healing ***(Stock et al., 1992).***

Bowman's layer:

It is a non-cellular membrane-like zone, 12 µm thick, detectable by light microscopy at the interface between the corneal epithelium and the stroma. It is well developed in humans and other higher mammals, but not well developed in lower mammals. It is formed of randomly arranged collagen fibers and proteoglycans. Collagen fibers in Bowman's layer are primarily of types I and III. The diameter of these fibers is 20-30 nm, which is smaller than that of the collagen fibers present in the corneal stroma ***(Fawcett, 1994).***

The collagen fibers in Bowman's layer are synthesized and secreted by the stromal keratocytes. So, continuity is observed between the collagen fibers in Bowman's layer and those in the stroma. Therefore, Bowman's layer is considered to be the anterior portion of the stroma ***(Nishida et al., 1988).***

Bowman's layer does not regenerate after injury; however, a normal epithelium is maintained in its absence. Furthermore, many mammals such as rabbits do not have Bowman's layer but still exhibit a well-organized epithelial structure that rests on a simple basal lamina. Therefore, the physiological role of Bowman's layer remains unclear ***(Wilson and Hong, 2000).***

Stroma:

The stroma constitutes about 90% of the cornea. Many characteristics of the cornea including its physical strength, stability of shape and transparency are attributed to the anatomic and biochemical properties of the stroma. The uniform arrangement and the continuous slow turnover of collagen fibers in the stroma are essential for corneal transparency. Both the epithelium and the endothelium play important roles in the maintenance of the biological activities of keratocytes as well as of the arrangement of collagen fibers through their regulation of the water content of the stroma. Thus, the epithelial cells, keratocytes and endothelial cells function in a coordinate manner to ensure corneal transparency ***(Robert et al., 2001).***

The corneal stroma consists of extracellular matrix, keratocytes (corneal fibroblasts) and nerve fibers. The cellular components occupy only 2-3% of the total volume of the stroma with the remaining portion comprising mostly the extracellular matrix collagen and glycosaminoglycans. Collagen (mostly type I) constitutes more than 70% of the dry weight of the cornea with smaller amounts of types III, V, and VI. The collagen fibers in the stroma are highly uniform in diameter (22.5-35 nm), and the distance between the fibers is also highly uniform. This regularity is a major determinant of corneal transparency. In the corneal stroma, the collagen fibers form about 300 lamellae. Each lamella courses parallel to the surface of the cornea from limbus to limbus. The turnover of the collagen molecules in the cornea is slow, requiring 2-3 years ***(Komai and Ushiki, 1991).***

Various glycosaminoglycans are present between collagen fibers in the corneal stroma, where they bind to the core proteins forming proteoglycans. The most abundant glycosaminoglycan in the cornea is keratan sulfate (65%). Others include chondroitin sulfate and dermatan sulfate. Glycosaminoglycans have the ability to absorb and retain large amounts of water. Although corneal hydration is regulated predominantly by endothelial pump, it is also influenced by the epithelial barrier, surface evaporation, intraocular pressure and stromal swelling pressure. The glycosaminoglycan content of the stroma plays a substantial role in this homeostatic process ***(Tanihara et al., 2002).***

The spindle shaped keratocytes are the predominant cellular components of the stroma and are thought to turn over every 2-3 years. They are scattered between the lamellae of the stroma and have long processes that are connected to the processes of the neighboring cells at their tips by gap junctions ***(Ueda et al., 1987).***

Keratocytes are similar to fibroblasts and possess an extensive intracellular cytoskeleton, including prominent actin filaments. This property allows the cell to contract and may be responsible for maintenance of corneal shape and for the packed structure of collagen in the stroma. The shape and the functions of keratocytes are regulated by the extracellular environment. Keratocytes in normal cornea are quiescent but are readily activated and become phagocytic by various types of insult to the stroma ***(Nishida et al., 1988).***

The activated keratocytes play an essential role in corneal wound healing after any injury to the stroma such as incisions and keratectomy. Keratocytes are also responsible for the