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Research Article

Cannabinoid Receptor CB1 Activation *In Vivo* Leads to Corneal Wound Healing After Chemical Injury, via Specific Receptor Vanilloid TRPV1 Desensitization

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Abstract

The clarity of vision depends on intactness of the cornea. Mechanical or chemical trauma, inflammation, refractive surgery, and infections result in corneal nerves disfunction and frequently induce corneal diseases such as opacities suffering from impaired vision. The aim of this study is to investigate the role of cannabinoids (CBs) in corneal nerves protection after induction of Corneal Epithelial Cells (CECs) damage by external noxious stimulus. CECs damage is induced *in vivo* using a concentrated solution of hydrogen peroxide on a model of Winstar rat. Results of our previous study report high density distribution of cannabinoid receptors CB1 in anterior eye, and high affinity ligand-receptor interaction. In this study we use preparation of instilled eyedrops of Arachidonoyl Ethanolamide (AEA), WIN55,212-2 (WIN) and AM251 to demonstrate the correlation between corneal CB1 receptors activation and corneal wound healing. The mechanism by which wound healing occurs is going to be demonstrate using transient receptor potential vanilloid TRPV1 antagonist Capsazepine (CPZ). All observations are made using slit-lamp.

Keywords: CannabinoidReceptors; Cornea; Endocannabinoids; Slit Lamp Observation; TRPV1 Receptor

Abbreviations

AA-5-HT	: N-arachidonoyl serotonin
cAMP	: cyclic adenosine monophosphate
CB1	: Cannabinoid receptor subtype 1
CB2	: Cannabinoid receptor subtipe 2
CBs	: Cannabinoids
CEC	: Corneal epithelial cell
DMSO	: Dimethyl sulfoxide
HCEC	: Human corneal epithelial cell
HP-β-CD	: Hydroxypropyl-β-cyclodextrin
PBS	: Phosphate buffered saline
PKA	: Protein kinase A
RPE	: Retinal pigment epithelium
SEM	: Standard error of the mean
TRPV1	: Transient receptor potential vanilloid subtype 1

Introduction

The eye is a complex organ that provides information on the form, light intensity, and color reflected from objects. It is divided into two segments: anterior and posterior. The anterior segment consists of the cornea, lens, iris and ciliary body, and the posterior segment consists mainly of the vitreous, retina and choroid. The

sclera and cornea constitute outer protective layers of the eye. The choroid, ciliary body and iris comprise the uveal tract. The choroid is a vascular layer that supplies the outer one third of the retina and the Retinal Pigment Epithelium (RPE). The ciliary body produces aqueous humor and regulates the contour of the cristalline lens. The retina is the neural sensory layer [1]. The focus in this article will be the cornea. This membrane forms the anterior 16% of the fibrous tunic of the eye. The principal feature of the cornea is its transparency, hence it is one of the most important dioptric means of the eye. The cornea consists of endothelium, Descemet's layer, stroma, Bowman's membrane and epithelium. The anterior surface is convex and directly related with external setting. The posterior surface is concave and confines anterior chamber of the eye. The outer side of the cornea has a slightly elliptical shape while the inner side has a round shape. Moreover the midst of the cornea is thinner than the borders. It is well known that the cornea is densely innervated [2]. Main thick stromal nerve bundles enter the cornea at the corneoscleral limbus and make midstromal plexus and dense Sub Epithelial Plexus (SEP), by repeatedly branching. Straight and curvilinear nerve fibers of the SEP penetrate Bowman's membrane and innervate corneal epithelium [3]. Corneal nerves respond to many sensations such as pain, temperature, or touch and functions in corneal reflex (blink) and tear production. Ocular surface, which consists of lacrimal film, cornea and aqueous humor, is directly exposed to external injury. Environmental stress induce inflammation. Chronic inflammation results in increased levels of proinflammatory cytokines, which propagates the inflammatory cycle by increasing oxidative and nitrosative stress, and stimulating the release of inflammatory mediators [4]. Corneal nerves contain many neurotrophic factors that are directly released from unmyelinated C fibers according to the inflammatory conditions and have functions to sustain the normal cornea and corneal wound healing (neurotrophic function) [5]. Mechanical or chemical trauma, inflammation, refractive surgery, and infections result in corneal nerves disfunction and frequently induce corneal diseases such as opacities suffering from impaired vision.

It has been suggested that cannabinoids have great therapeutic potential in inflammatory processes [6]. There are at least two tipes of cannabinoid receptors, CB1 and CB2. CB1 and CB2 receptors activate intracellular G-proteins, which transduce signal to a variety of effectors, such as ion channels, adenylyl cyclase, phospholipase C and the mitogen-activated protein kinase cascade [7].

Straiker used a subtype-specific affinity-purified polyclonal abtibody to the CB1 receptor to determine its localization within the human eye. Results report that CB1 labeling was detected in several locations in the anterior segment of the eye. Strong labeling was detected in corneal epithelium, ciliary epithelium, ciliary muscle and in the blood vessels of the ciliary body. CB1 receptors are also expressed in the sphincter papillae and trabecular meshwork. Moreover CB1-like immunoreactivity (CB1-LI) has been found in corneal nerves. In the retina CB1 receptors has been found on the two synaptic layers, in the outer and inner plexiform layers and in the ganglion cell layer [8].

In our previous studies (unpublished data) data on CB1 receptor corneal distribution and ligand affinity were obtained performing *in vitro* binding assays, on membrane preparation from isolated corneal epithelial cells of Winstar rats, using the antagonist [^{3}H]SR141716A and the agonist [^{3}H]WIN55,212-2. Values reported were Bmax 4,82±0,84 pmol/mg and kd 0,324±0,17 nM for SR141716A. For WIN55,212-2 Bmax is 3,80±0,32 pmol/mg and kd is 3,26±0,18 nM. CB1 receptors belong to the superfamily of seven transmembrane-spanning domain, G protein-coupled receptors [8] and are negatively coupled to adenylyl cyclase [9] and Ca^{2+} channels [10,11] and positively coupled to K^{+} channels [12,11]. There are two types of Ca^{2+} channels (i.e., voltage-dependent and receptor-operated channels). The Transient Receptor Potential (TRP) channels are of the latter type. TRP channels can be classified into six main subfamilies: the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) groups. TRP channels are expressed in almost every tissue and cell type and display an important role in the regulation of various cell functions [13].

Transient Receptor Potential Vanilloid receptor subtype 1 (TRPV1) is expressed in the cornea, on the ophtalmic branch of trigeminal nerve endings. They are activated by injury to epithelial cells resulting in endogenous ligand release, environmental stresses and infection leading to increases in proinflammatory cytokine and chemoattractant expression [14-17]. In many tissues, TRPV1 and CB1 are coexpressed and functionally interact with one another. Such is the case in the colonic epithelium, in neuronal enriched mesencephalic cultures, primary sensory neurons and myometrial smooth muscle cells [18-21]. In 2013 Yang use immunocytochemistry to determine CB1 and TRPV1 colocalization in Human Corneal Epithelial Cells (HCEC) [22].

It is well known that there is a biofunctional correlation between CB1 activation induced-responces and TRPV1 pathway. Nociceptive stimuli activate TRPV1 and induce proinflammaory cytokine release [23]. CB1 activation decreases the release of cytokines induced by TRPV1 activation [16]. Such suppression occurs through protein-protein interaction between TRPV1 and CB1. The activity of TRPV1 both in physiological and pathological conditions is regulated by a series of intracellular signalling molecules. Among those, the adenylyl cyclase -cyclic AMP (cAMP)- Protein Kinase A (PKA) pathway seems to have a particular importance [24-28]. In inflammatory conditions various agents induce PKA activity that results in sensitization of TRPV1-mediated responses [29-31]. This findings suggest that TRPV1 desensitization occurs through Gi/Go-protein-coupled cannabinoid 1 (CB1) receptor activation and PKA activity reduction, leading to declines in TRPV1 phosphorylation status (Figure 1).

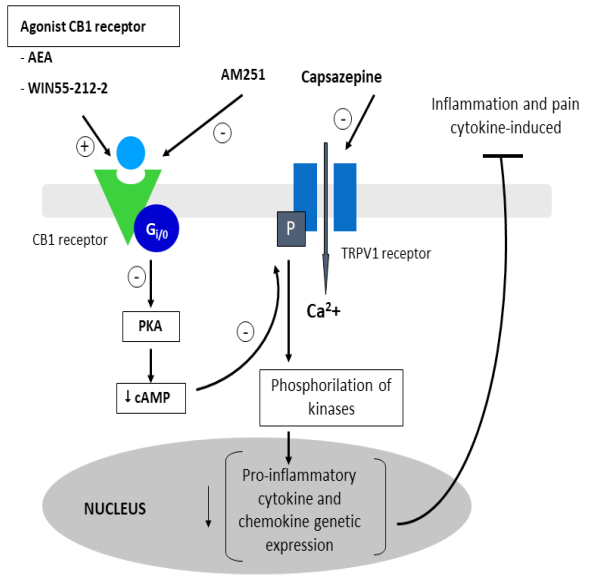

Fig. 1

Figure 1: CB1 mediated responces pathway. The figure shows cannabinoid receptor CB1 (Gi/Go protein coupled receptor) activation and subsequent [cAMP] reduction, via PKA inhibition. This prevents transient receptor vanilloid TRPV1 phosphorylation and calcium influx. The result of this inhibition is the reduction of pro-inflammatory cytokines level.

In this study we investigate the role of cannabinoids in preservation of anterior eye integrity through protection of corneal nerves from excitotoxicity.

We induce corneal injury *in vivo* by application on Winstar rat models of concentrated Hydrogen Peroxide solution. In order to demonstrate the involvement of CB1 corneal receptor in corneal wound healing via TRPV1 activation we use non selective syntetic Analogue of Endocannabinoid Anandamide (AEA), CB1/CB2 partial agonist WIN55,212-2 (WIN), selective CB1 antagonist AM251 and the TRPV1 antagonist Capsazepine (CPZ).

Materials and Methods

Chemicals

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD, Encapsin®, molecular weight [mw] = 1297.4, degree of molar substitution 0.4) was obtained from Sigma-Aldrich (Milan, Italy). Arachidonoyl ethanolamide was purchased from Organix Inc. (Woburn, MA, USA). The synthetic cannabinoid receptor agonist WIN55212-2 was purchased from Tocris Cookson Ltd. (Bristol, United Kingdom). The CB1 receptor antagonist AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) was purchased from Tocris Cookson Ltd. (Bristol, United Kingdom).The selective TRPV1 channel antagonist Capsazepine was purchased from Sigma-Aldrich (Milan, Italy).

Ophthalmic Preparations Formulation

An ethanol solution of anandamide was evaporated under a stream of nitrogen, and the compound was redissolved in an aqueous 45% 2-hydroxylpropyl-β-cyclodextrin solution. Solution pH was then adjusted to 7,4 with NaOH and. Final concentration of anandamide was 2,5 mg/ml (1 drop, 25 µL dose = 62,5 µg). WIN55,212-2 was dissolved in a vehicle solution containing 500 mL of 150 mM NaCl and 45% 2-HP-β-CD, and then diluted with sterile saline. Final concentration of WIN55212-2 was 2 mg/ml (1 drop, 25 µL dose = 50 µg). The CB1 receptor antagonist AM251 was dissolved in 45% HP-β-CD. Solution pH was adjusted to 7,4 with NaOH. Final concentration of AM251 was 0.5 mg/mL.The TRPV1 antagonist capsazepine was diluted in PBS 50 mM and dissolved in DMSO. The pH was adjusted to 7.4 with NaOH and the solution was made isotonic with sodium chloride. Final concentration of CPZ was 0,37 mg/mL (1 drop, 25 µL dose = 9,25 µg).

Characteristic of opthalmic preparations

A 713 pH Meter (Metrohm, Herisau, Switzerland), equipped with a combined Ag/AgCl glass electrode was used. The pH measurements were performed in triplicate at 25˚C. Osmotic activities were analyzed by using an automatic cryoscopic osmometer (Osmomat 030-D Gonotech, GmbH, Berlin, Germany). Before the analyses, the osmometer was calibrated with 300 mOsm NaCl standard and ultrapure bidistilled water. The measurements were made in triplicate at 25˚C.

Animals

Twentysix male Wistar rats (Harlan, S. Pietro al Natisone (UD), Italy) weighting 200-220 g (seven-eight weeks) were used in this study. The rats were housed for one week in paired cage at light/darkness cycles of 12 h and with free access to food and beverage. All experiments were performed in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian law on Animal Care No. 116/1992 and the Directive 2010/63/EU. The protocols were approved by the local Animal Care and Use Committee of the University of Bari. All efforts were made to reduce the number of animals used.

Slit lamp

Slit lamp is an optical, non invasive, instrument used to examine *in vivo* anterior segment and posterior segment of the eye. In this instrument there are four sistems: 1)lightening sistem; 2)observing sistem; 3)microscope; 4)mechanic sistem. The lightening sistem is made by an high-intensity light source and a lens sistem that focuse the light beam in a restricted area to allow this to pass through eye's tranparent structures. This is important to evaluate physiological and pathological structural features of the eye, trading on Tyndall effect. In ophtalmology Tyndall effect occurs because of the pathological manifestation of corpuscols (inflammatory cells) in the aqueous humor of the anterior chamber

of the eye.

Corneal Injury Induced by Topic Application of Hydrogen Peroxide Solution

In this study we use topical application of hydrogen peroxide solution (H_2O_2) (Figure 2). The observation of concentration-dependent effects has been obtained using three different concentrations: 150 µM, 200 µM and 300 µM. We use a starting 3% hydrogen peroxide solution, the lowest commercially available concentration. On this we take 172 µL, 230 µL and 350 µL and made a dilution in 1L to obtain final solutions respectively 150 µM, 200 µM and 300 µM H_2O_2. This solutions are ipotonic. Indeed we estimate isotonic a 1% H_2O_2 solution with an osmometer. To obtain a isoosmotic solution we add a pH 7,4 phosphate buffer. To obtain statistically relevant data we used 8 rats, divided into two groups of 4 specimens (Table 1). In the first group Left Eye (LE) has been treated with 150 µM H_2O_2, while Right Eye (RE) work as control (physiological solution, NaCl 0,9%). In the second group we have administered 200 µM H_2O_2 solution to RE and 300 µM H_2O_2 to LE. Treatement consists on topical administration of eyedrops (two drops: volume dose = 50 µL). Observation has been made using slit lamp.

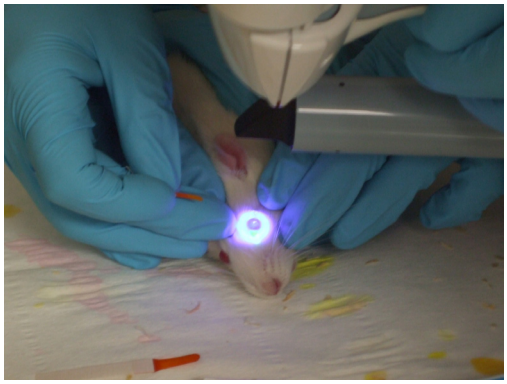

Figure 2: Topical Treatment. The figure shows a Winstar rat during hydrogen peroxide solution eyedrops application, in order to obtain corneal chemical injury. The upper eyelid is slightly pulled away from the globe during instillation of two drops (50 µL) of solution.

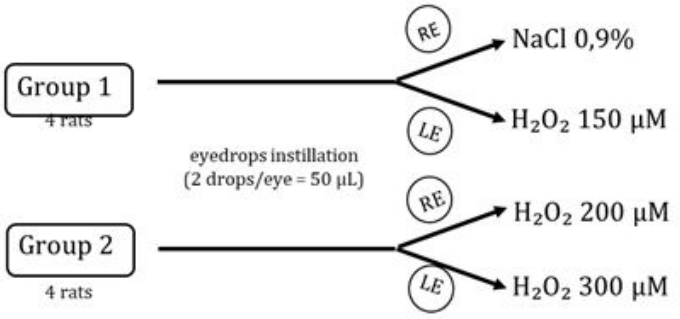

Table 1: Experimental Protocol For Chemical Injury Induction. Abbreviations- RE : right eye; LE : left eye.

Corneal Wound Healing After Topical Administration of Cannabinoid Compounds

We study the effect of cannabinoids on corneal wound healing using the synthetic analogue of endocannabinoid Arachidonoyl ethanolamide (AEA), and sinthetic experimental compounds: WIN55,212-2, a non selective cannabinoids receptor agonist, and AM251, a selective CB1 receptor antagonist. To demonstrate a possible mechanism via TRPV1 desensitization after CB1 activation we use a selective calcium channel TRPV1 blocker: Capsazepine (CPZ). Topical application would be the ideal form of administration to minimise possible systemic adverse side effects and maximise the dose at the site of action. Natural cannabinoids as well as synthetic forms are highly lipophilic. Cyclodextrins are well known for their ability to increase the aqueous solubility and stability of many lipophilic drugs. The low aqueous solubility has been overcome through the use of 45% 2-hydroxylpropyl-β-cyclodextrin. To obtain statistically relevant data we used 18 rats previously exposed to oxidative stress, divided into three groups of 6 specimens, treated with instilled eyedrops of different solutions (Table 2). Each group has been subjected to experimentation for 10 days. Corneal injury was obtained by pretreatment with 200 µM H_2O_2 solution. The first group has been trated with physiological solution (NaCl 0,9%), topically applied (two drops = 50 µL), bilaterally, two times/day, for 10 days. In the second group right eye (RE) of each rat has been treated with two drops (50 µL) of AEA 2,5 mg/mL (7,2 mM) two times/day for 10 days. During instillation, the upper eyelid was slightly pulled away from the globe. In the left eye (LE) we administered AEA 2,5 mg/mL at 15 minutes after pretretment with eyedrops of TRPV1 selective antagonist capsazepine 0,37 mg/mL (1 mM solution). In the third group RE has been treated with two drops of WIN55,212-2 2 mg/mL (4,7 mM) two times/day for 10 days. In the LE we administered WIN55,212-2 at 15 minutes after pretreatment with eyedrops of selective CB1 antagonist AMG251 0,5 mg/mL (0,9 mM solution).

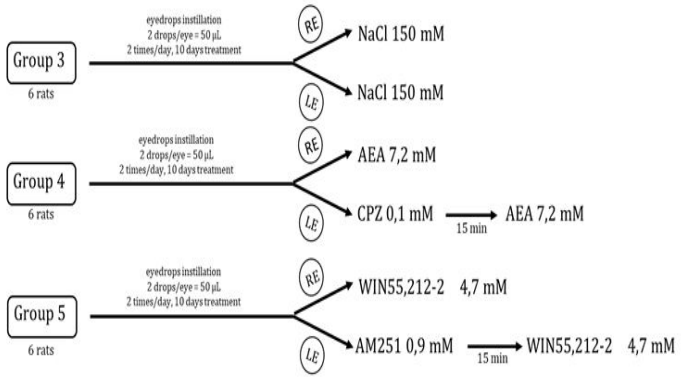

Table 2: Experimental Protocol For Ophthalmic Treatment. Abbreviations- AEA**:** Arachidonoyl ethanolamide; CPZ: Capsazepine; LE**:** left eye; RE:

right eye.

Data Anlysis

Data analysis was performed using statistical software (GraphPad Prism Software, version 5.0). Significance between mean were evaluated by student t-test ($p < 0,05$ (*); $p < 0,001$ (**)). Significance within and between data groups was evaluated by ONE WAY ANOVA and the Bonferroni's test for the evaluation of of the effects a specific data group on variance ($p < 0,05$ (*); $p < 0,001$ (**), as in figures). All data are presented as mean±S.E.M.

Results

Slit lamp observation on corneal injury

Corneal epithelium has been treated with fluorescein to observe areas of opacification in which oxidative stress causes corneal trasparency and integrity impairment (Figure 3).

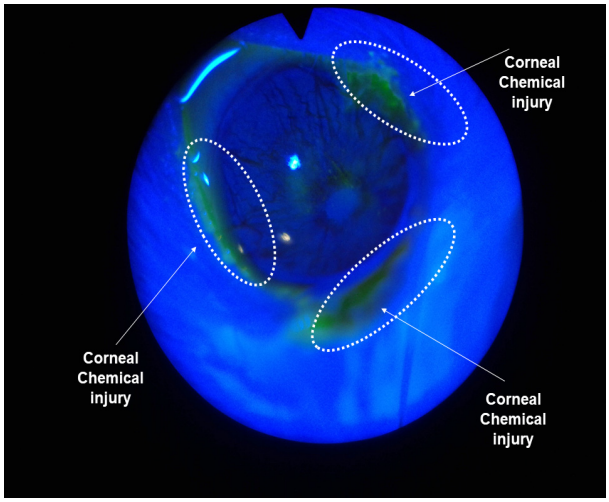

Figure 3: Slit lamp observation The figure shows areas of opacification in a Winstar rat's cornea due to the presence of inflammatory cells (corpuscols) in the aqueous humor of the anterior chamber of the eye. This result comes from *in vivo* observation with slit lamp at 40 minutes after H_2O_2 200 µM solution topical application (50 µL). Corneal epithelium has been treated with Fluorescein. Impaired areas are highlighted in the figure with white shapes.

Damage is expressed as percentage of surface area, referred to control (physiological solution, corneal injury 0%), controlateral eye, using a colorimetric and topographic scale of corneal injury (Figure 4), graphically correlated to observation time (Figure 5). Percentage of surface area damaged has been calculated as mean value of n (n = 4) observations. To every percentage is related a S.E.M. to define the range of dispersion of the set of data values.

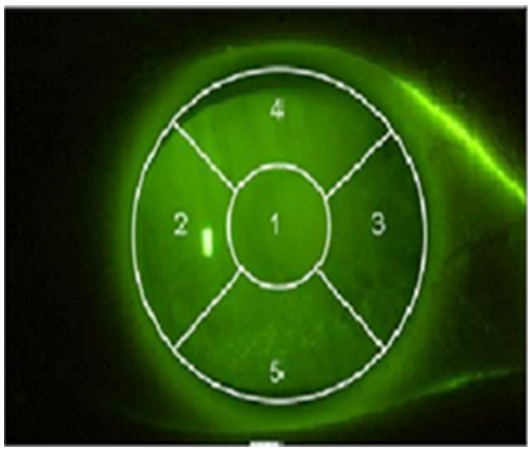

Figure 4: Colorimetric And Topographic Scale of Corneal Injury. The figure shows a colorimetric and topographic scale of corneal injury in a model of Winstar rat, after coloration with Fluorescein. Values, from 1 to 4, by which corneal damage after oxidazing solution topical application is expressed, are referred to different type (1: micropunctate; 2: macropunctate; 3: coalescent macropunctate; 4: patch), surface area (1: 1-15%; 2: 16-30%; 3: 31-45%; 4: >45%) and density (1; very slight; 2: slight; 3: moderate; 4: severe) of damage.

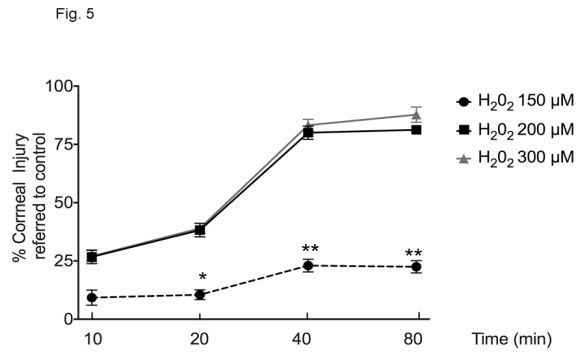

Figure 5: The graphic shows percentage of corneal chemical injury, obtained by topical application of H_2O_2 solutions at different concentrations (150 µM, 200 µM, 300 µM), referred to control (physiologic solution eyedrops, NaCl 0,9%, corneal injury 0%), expressed as rate of surface area damaged. Corneal injury is graphically correlated to observation time (10 minutes, 20 minutes, 40 minutes, 80 minutes). (n = 4); $p < 0,001$ (**); $p < 0,005$ (*).

First analysis demonstrates time dependent effect after hydrogen peroxide topical application (Figure 6). Observation has

been made at regular time intervals: 10 min, 20 min, 40 min, 80 min and we found that only after 40 minutes cornea is dramatically damaged (surface area >45%, corresponding to severe density).

Second analysis demonstrates concentration dependent effect after hydrogen peroxide topical application (Figure 7). We use three different concentrations: 150 µM (group 1 = 4 rats, LE), 200 µM (group 2 = 4 rats. RE), 300 µM (group 2 = 4 rats, LE) . Observation at 40 minutes after eyedrops application reports that 150 µM solution causes a slight density injury (surface area 16-30%). We estimate that 200 µM solution causes severe density damage (at 40 minutes 80±5,71% of corneal surface damageded). There's no statistically relevant difference between 200 µM and 300 µM solutions effects.

Fig. 6

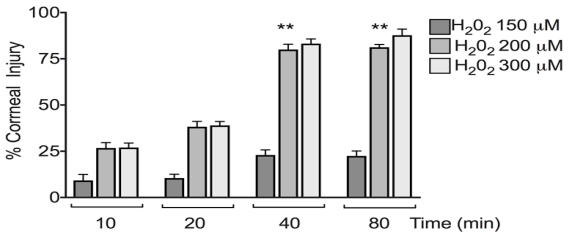

Figure 6: The histogram reports data obtained from time-dependent effects analysis. Observations on corneal injury have been made with slit lamp at 10 minutes, 20 minutes, 40 minutes and 80 minutes after H_2O_2 150 µM, 200 µM and 300 µM topical application and coloration with Fluorescein. Each data group of different concentration solutions is referred to a specific time of observation. There's no relevant rise of damage up to 40 minutes (surface area < 45%). There's no statistically relevant difference between 40 minutes and 80 minutes observations. (n = 4); p < 0,001 (**)

Fig. 7

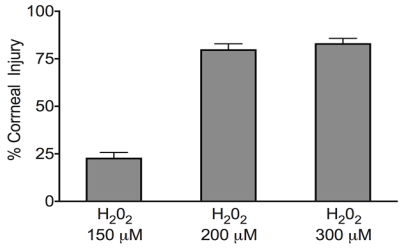

Figure 7: The histogram reports data obtained from concentration-dependent effects analysis. Observations on corneal injury have been made with slit lamp at 40 minutes after H_2O_2 150 µM, 200 µM and 300 µM solutions eyedrops application and coloration with Fluorescein. $H_2O_2$150 µM causes not relevant injury (surface area damged 23±5,48%, slight density). H_2O_2 200 µM causes severe density damamge (surface area ≥ 45%). There's no statistically relevant difference between 200 µM and 300 µM solutions (n = 4).

Slit Lamp Observation On Corneal Wound Healing

Corneal epithelium has been treated with fluorescein to observe areas of opacification. Damage is expressed as rate of control (H_2O_2 200 µM, 40 minutes, corneal injury 100%) controlateral eye, referring to colorimetric and topographic scale of corneal injury (Figure 4), graphically correlated to observation time (Figure 8). Percentage of surface area damaged has been calculated as mean value of n (n = 6) observations. To every percentage is related a S.E.M. to define the range of dispersion of the set of data values. Observation has been made at day 3, day 5 and day 10 (Figure 9,Figure10). Results of observations are reported in (table 3).

Fig. 8

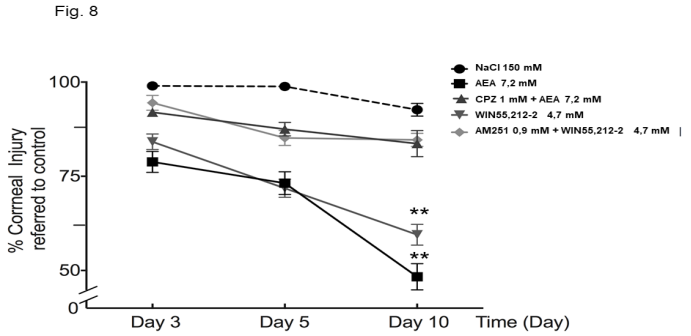

Figure 8: The graphic shows percentage of corneal chemical injury after treatment with different ophthalmic preparations (NaCl 150 mM; AEA 7,2 mM; CPZ 1 mM + AEA 7,2 mM; WIN55,212-2 (WIN) 4,7 mM; AM251 0,9 mM + WIN55,212-2 (WIN) 4,7 mM) on chemical damaged Winstar rat's corneas, referred to control (H_2O_2 200 µM, time of observation 40 minutes, corneal injury 100%), expressed as rate of surface area damaged. Corneal injury is graphically correlated to observation time (day 3, day 5, day 10). (n = 6); p < 0,001 (**).

Fig. 9

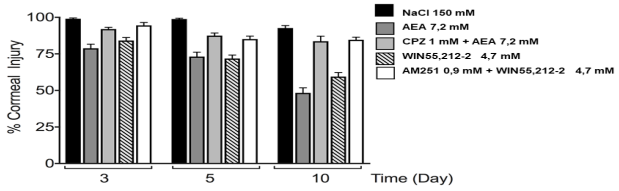

Figure 9: The histogram reports data obtained from time-dependent analysis on corneal wound healing improvement. Observations *in vivo* have been made with slit lamp at day , day 5 and day 10, after coloration of corneal epithelium with Fluorescein, during treatment with ophthalmic preparations of NaCl 15 mM, AEA 7,2 mM, CPZ 1 mM + AEA 7,2 mM, WIN55,212-2 (WIN) 4,7 mM, AM251 0,9 mM + WIN55,212-2 4,7 mM, on injured Winstar rat's corneas (n = 6).

Fig. 10

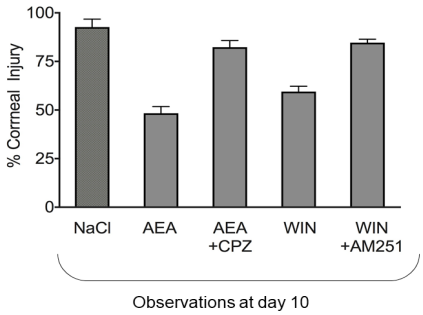

Figure 10: The histogram reports data obtained from observations on corneal wound healing at day 10 after topical treatment with differents ophthalmic preparations (NaCl 150 mM; AEA 7,2 mM; CPZ 1 mM + AEA 7,2 mM; WIN55,212-2 4,7 mM; AM251 0,9 mM + WIN55,212-2 4,7 mM) on injured Winstar rat's corneas. Each observation has been made with slit lamp, after coloration with Fluorescein. Compared to control (H_2O_2 200 µM, 40 minutes, severe density, surface area damaged 100%), treatment with AEA 7,2 mM and WIN55,212-2 (WIN) 4,7 mM causes relevant corneal wound healing (AEA = 48,33 ± 3,46 %; WIN55,212-2 = 59,50 ± 2,74 %). Loss of efficacy is observed with pretreatment with Capsazepine 1 mM and AM251 0,9 mM (CPZ + AEA = 83,67 ± 3,46 %; AM251 + WIN55,212-2 = 84,67 ± 1,74 %).

Corneal injury in RE of group 3 (physiological solution, NaCl 0,9%) is 99±0,52% compared to the control at day 3, 98,83±0,53% at day 5 and 92,66±1,70% at day 10.

Corneal injury in RE of group 4 (AEA) is 78,83 ± 2,80 % compared to the control at day 3, 73,17 ± 3,02 % at day 5 and 48,33 ± 3,46 % at day 10. In LE of the same group (CPZ + AEA) corneal surface area damaged raises, due to the presence of TRPV1 antagonist, at 92,00 ± 1,15 % after 3 days, 87,50 ± 1,77 % after 5 days and 83,67 ± 3,46 % after 10 days. In our previous studies (unpublished data) we used N-arachidonoyl serotonin (AA-5-HT) with anandamide (AEA) instead of capsazepine (CPZ) with AEA. Results from these experiments demonstate non statistically relevant difference betwwen percentage of corneal injury observed with two diffent TRPV1 antagonists.

Corneal injury in RE of group 5 (WIN55212-2) is 84,17 ± 2,06 % compared to the control at day 3, 71,83 ± 2,40 % at day 5 and 59,50 ± 2,74 % at day 10. In LE of the same group (AM251 + WIN55,212-2) corneal surface area damaged raises, due to the presence of selective CB1 antagonist, at 94,50 ± 1,98 % after 3 days, 85,17 ± 1,99 % after 5 days and 84,67 ± 1,74 % after 10 days.

	Ophthalmic treatments									
	NaCl 150 mM		AEA 7,2 mM		CPZ 1mM + AEA 7,2 mM		WIN 4,7 mM		AM251 0,9 mM + WIN 4,7 mM	
	Corneal injury mean ± S.E.M. (%)	n	Corneal injury mean ± S.E.M. (%)	n	Corneal injury mean ± S.E.M. (%)	n	Corneal injury mean ± S.E.M. (%)	n	Corneal injury mean ± S.E.M. (%)	n
Day 3	99,00 ± 0,52	6	78,83 ± 2,80	6	92,00 ± 1,15	6	84,17 ± 2,06	6	94,50 ± 1,98	6
Day 5	98,83 ± 0,53	6	73,17 ± 3,02	6	87,50 ± 1,77	6	71,83 ± 2,40	6	85,17 ± 1,99	6
Day 10	92,67 ± 1,69	6	48,33 ± 3,46	6	83,67 ± 3,46	6	59,50 ± 2,74	6	84,67 ± 1,74	6

Table 3: Results from *in vivo* observations of corneal wound healing. Abbreviations- AEA : Arachidonoyl ethanolamide; CPZ : Capsazepine; SEM : Standard error of the mean;WIN : WIN55,212-2.

Discussion

Cornea is the most external structure of the eye and so it is exposed to many external noxious agents. Visual clarity depends on intactness of this barrier. Epidemiologic finds report an high percentage of work accidents in metallurgic sector compromising vision. Another case of corneal injury is olive leaves wound, extreamly common in south of Italy during autumn season. Furthermore environmental stresses, such as UV radiation and air pollution, lead to cell damage through production of Reactive Oxygen Species. Refractive surgery also leads to corneal injury.

The cornea is densely innervated by small diameter myelinated fibers originating from the ophtalmic division of the trigeminal nerve [32-35], contributing to mantain ocular homeostasis and to support the integrity of the anterior eye through trophic influences [36-38]. Mechanical or chemical trauma, inflammation, refractive surgery, and infections result in corneal nerves disfunction and frequently induce corneal diseases such as opacities suffering from impaired vision. Injury occurs through release of inflammatory mediators from sensory nerves so it is relevant to identify novel strategies to prevent this pathologic response from becaming chronic. Previous study demonstrate neuroprotective role of cannabinoids in excitotoxicity models [39-41] and CB1 expression was detected in the corneas of isolated human eyes [8]. The cannabinoid receptor subtype 1 (CB1) modulates, through the GTP binding protein (Gi), a number of

important physiological processes [42], such as neuroprotection. The signal transduction mechanism which mediate this biological action is not well characterized. In this study we have investigated the effects of 10 days treatment with eyedrops of arachidonoyl ethanolamide (AEA) on *in vivo* Winstar rats models.

Data obtained from observation with slit lamp report a great reduction of corneal injury at day 10. Percentage of corneal surface damaged is 48,33 ± 3,46 %, compared with non treated eyes (group 3, physiological solution) in which corneal injury is 92,67 ± 1,69 % at day 10. This value (92,67 ± 1,69 %) lower than 100%, can be intended as physiological response to injury. AEA behaves as a partial cannabinoid receptor agonist with less CB2 than CB1 efficacy. AEA activates vanilloid (TRPV1) receptors in addition to CB1 and CB2 receptors and there is also growing evidence for the existence of non-CB1, non CB2, non-vanilloid pharmacological targets for AEA [43]. Indeed to demonstrate the correlation between corneal wound healing and cannabinoid receptor activation it has been required to use the synthetic compound WIN55,212-2. This is an established cannabinoid agonist which has marginally grater CB2 than CB1 affinity [7]. The loss of protective effect against oxidative stress on the cornea, because of the presence of AM251, a selective CB1 antagonist, demonstrates that corneal wound healing occurs through activation of CB1 receptors. Many authors report that in other tissues TRPV1 and CB1 are coexpressed and functionally interact with one another. In 2013 Yang use immunocytochemistry to determine CB1 and TRPV1 colocalization in Human Corneal Epithelial Cells (HCEC) [22]. TRPV1 channel contributes to the secretion of inflammatory mediators in the corneal epithelium [44].

The coexpression of TRPV1 and CB1 in the corneal epithelium prompted us to demonstrate that CB1 activation reduce TRPV1-induced inflammtory responces. This pathway has been validated in this study by showing that pretreatment with the selective TRPV1 antagonist capsazepine (CPZ) nearly fully attenuate AEA effects. The use of N-arachidonoyl serotonin (AA-5-HT), selective TRPV1 antagonist and specific calcium currents TRPV1-dependent blocker, did not produce statistically relevant results compared to the TRPV1 antagonist capsazepine.

Taken together, these results suggest that cannabinoid receptor CB1 is a potential drug target to improve the outcome of corneal wound healing in inflammatory process that occurs subsequently to chemical or mechanical injury, in neurodegenerative diseases and in many other pathological states. Amongst the others diseases affecting the cornea, that may cause blurred or irregular vision, up to total blindness, there are keratoconus, keratitis, corneal ulcer, scarred cornea (macula). In this contest a treatment with hypothetical esocannabinoids drugs can result useful to promote a reductive modulation of corneal injury pathological status and to prevent degeneration that, if neglected, could lead to blindness.

Conclusion

This study reports improvement of corneal wound healing, in a model of Winstar rat exposed to chemical injury, with ophthalmic treatment with synthetic analogue of Arachidonoyl ethanolamide. Experiments demonstate the involvement of CB1 activation and TRPV1 desensitization, with subsequent reduction of pro-inflammatory mediators cellular level. After a 10 days treatment percentage of corneal surface damaged is only 48,33±3,46% instead of 92,67±1,69% in non treated eyes. This important result prompts us to hypothesize the formulation of ophthalmic preparation of esocannabinoids to regain or to maintain corneal integrity in many phatological states that involve inflammatory processes. Chronicity of corneal inflammatory state, with loss of transaparency and impaired vision, leads to blindness. Thus, the possibility of topic treatment, which is an ideal form of administration to minimise possible systemic adverse side effects and maximise the dose at the site of action as demonstrated in previous studies [45], to repair corneal injury is a great prospective in ophthalmology.

Author Contributions

MD Lograno elaorated the hypotesis and theory, designed the studies and analyzed the results. V Alicchio conducted the experiments and wrote the manuscript. The two authors approved the manuscript.

Aknowledments

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Conflict of Interest

The authors have no conflict of interest or finanacial relationship related to this manuscript.

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