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Is tacrolimus effective against choroidal neovascularization? A preliminary study

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Abstract

To evaluate the effectiveness of tacrolimus used topically in inhibiting a rat model of laser-induced choroidal neovascularization. Thirty-six Long-Evans pigmented rats were utilized in this paper. Bursting lesions are performed on the retina of one eye in each animal to induce choroidal neovascularization (CNV) by argon laser. The animals were separated into 3 groups of 36 rats. Twelve animals in each group and one eye per animal were topically administrated daily for 5 weeks at 0.3%, 0.5% tacrolimus, and saline respectively. Fluorescein angiography (FA) was performed in groups 1, 2, and 3 (control group) in the 3rd week, 4th week, and 5th week after the initial retinal photocoagulation. FA is graded for each of the three lesions as follows: score 0: no leakage, score 1: slight leakage, score 2: moderately leakage, and score 3: strong leakage. At the end of the study, the eyes were enucleated and prepared for histological examination. According to fluorescein angiography (FA) score comparisons, in group 1 (topical 0.3% Tacrolimus) there was only a significant difference between its controls at the 5th week after retinal photocoagulation (p=0.05). In group 2 (topical 0.5 % Tacrolimus) there was a significant difference when compared to control eyes in the 4th week and the 5th week (p=0.018, p=0.021). Topical instillation of tacrolimus at a concentration of 0.5% following the laser photocoagulation significantly reduced leakage of CNV versus controls in the 4th and the 5th weeks, the time points tested. Tacrolimus at a concentration of 0.3 % significantly reduced CNV only 5 weeks after retinal photocoagulation. These results show that there is a dose-response of topical tacrolimus on the suppression of CNV. Tacrolimus is a well-penetrating drug through the cornea and following topical administration, can reach effective concentrations depending on application way and dose. This study needs to be performed in other experimental models and then clinical trials.

Keywords: Choroidal neovascularization, tacrolimus, argon laser, fundus angiography

Introduction

Choroidal neovascularization can consist of many chorioretinal diseases, which appear often in age-related macular degeneration (ARMD), indicating forming of new blood vessels may be both subretinal pigment epithelium or subretinal space, or one [1-3].

Neovascular ARMD has been shown to be one of the most common causes of irreversible vision loss in developed countries [4-6]. The prevalence of ARMD has been demonstrated to be 7.3% and 12.3% in the Asian and European populations, respectively [4].

CNV involves several degrees of inflammatory components depending on the underlying disease and stage of CNV development (initiation, inflammatory active, inflammatory inactive) [7]. Novel pharmacological research has focused on interrupting these steps within the dynamic evolution. Treatment options for this disorder include macular photocoagulation, photodynamic therapy (PDT), submacular surgery, macular translocation, ionizing radiation, and antiangiogenic therapies such as Pegaptanib, Bevacizumab, Ranibizumab, Aflibercept, and Brolucizumab [8-15].

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Tacrolimus, known as FK506, is a neutral macrolide compound isolated from a strain of soil fungus, Streptomyces tsukubaensis [16]. It is an immunomodulatory agent that inhibits the calcineurin enzyme through T-cell activity [17]. It was first discovered as a macrolide immunosuppressive [18]. Tacrolimus has been promisingly used for the prolongation of graft survey in transplant patients and refractory uveitis including Behçet's disease, however, it may cause severe adverse effects as administrated systemically [19-22].

Most of the eye diseases in the anterior segment such as uveitis, and open-angle glaucoma can be treated topically by administrating medication in the appropriate form or vehicle. It is also used in severe dry eye and allergic conjunctivitis, and in the prevention of rejection after corneal transplantation [23-25]. Although it has usually been considered that topical administration does not let enough drug concentrations attain in the posterior portion of the eye to treat retinal diseases, some previous studies it has shown that various topical applications of tacrolimus can penetrate the cornea and sustain its concentration at aqueous humor, vitreous, sclera. Although topical tacrolimus has side effects such as temporary ocular irritation, it is generally well tolerated [24,26-28].

Therefore, in the current work, we evaluated the impact of tacrolimus applied topically on experimental laser-induced choroidal neovascularization in rats.

Material and Methods

Animals

The 36 male Long Evans pigmented rats utilized in this research, which ranged in weight from 200 to 250 gr, were handled strictly under the resolution of the Association for Research in Vision and Ophthalmology (ARVO). For the experiment, only one eye was used. The ethics committee at Tulane University in the United States gave its approval, and the study was carried out in the ophthalmology research laboratory. A cover slip (24 x 60-1 mm No. 1) and 2.5% hydroxypropyl methylcellulose solution were used as a contact lens to observe the fundus in all rats both before and after laser therapy. Animals that had previously displayed media opacity or retinal injury were not included in the study. Additionally animals were selected from the same age group.The rats were given intraperitoneal injections of 13.0 mg/ml xylazine and 94.7 mg/ml ketamine to put them to sleep before all procedures.

Experimental Laser-Induced Subretinal Neovascularization

2.5% phenylephrine and 1.0% tropicamide were used to dilate the pupils, while 0.5% proparacaine was used as a topical anesthetic. A photocoagulator's slit-lamp delivery method was used to deliver an argon ion laser (488.0-528.7 nm), with a portable coverslip (24x60-1 mm No. 1) acting as a contact lens. The laser's output was 0.42 W, its spot size was 50 microns, and its pulse duration was 0.05 seconds. Every animal undergoes bursting lesions on the retina of one eye to cause choroidal neovascularization (CNV) using an argon ion laser. In each example, three lesions were independently formed around the optic nerve, each about one burn diameter from the head of the optic nerve (12, 1:30, and 3 O'clock positions). Burn lesion formation over significant vessels was also prevented. Bruch's membrane rupture was verified by the bubble that formed during laser treatment [29]. Animals that exhibited following laser treatment intensive retina and vitreous hemorrhage were substituted. The rats were randomly separated into 3 groups of 36 animals: Twelve rats in each group were topically administrated daily three times as one drop of solution to each eye for 5 weeks at 0.3%, 0.5% Tacrolimus (Prograph), and saline (Group 1, 2, and 3 respectively). In all groups, the rats were administered the drug promptly following retinal laser burns.

CNV Grading with the Help of Fluorescein Angiography

Fluorescein angiography was taken in every eye before topical application of tacrolimus to confirm the presence of CNV; it was repeated in all groups in 3rd week and 5th week after created burns. Sodium fluorescein (AK-FLUOR 10%, Akorn, Inc., Buffalo Grove, IL) (0.1-0.2ml) was injected intraperitoneally.

Two investigators graded the amount of leaking in late-phase fluorescein angiography (100-150 seconds after dye injection) in a concealed manner to assess drug treatment. Scores for angiograms were given as follows: 0, no leaking; 1, slightly leaked; 2, moderately leaked; and 3, strongly leaked [30,31]. When two scores given to the same lesion did not overlap, the higher score was utilized for the analysis. The experimental CNV time course features fluorescein leakage culminates, which can be seen 2 to 5 weeks after laser induction, hence additional examination was skipped. After that, fluorescein leakage diminishes, though CNV is still histologically detectable [30,31]. Six rats from each group were slaughtered four and five weeks after receiving a laser burn in all groups. Inhaled carbon dioxide was used to euthanize the animals, as they were in strong anesthesia.

Histological Evaluation

The eyes were immediately removed, and a fixative containing 2% paraformaldehyde and 3% glutaraldehyde was used to prepare them for histological investigation. The globes were sliced for dehydration after 48 hours of fixing. Technovit 7100 was infiltrated overnight into the eyes after pre-infiltration with ethanol and Technovit 7100. The tissues were sliced at 3 microns, immersed in methacrylate overnight, and stained for light microscopy by using 1% toluidine blue. We only assessed lesions that had decent serial segments through the center.

The control and treatment eyes were investigated for the presence of fibrovascular proliferation emerging through the broken RPE, Bruch's membrane, and the retina. The existence of CNV was described by CNV extending into the lesion, CNV membranes, and new vessels in the subretinal space. The eyes were given a grade of 0 or 1 based on whether there was evidence of fibrovascular growth or not.

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Statistical Analysis

The Mann-Whitney U analysis was performed to assess the fluorescence in the 4 groups during angiography, and Fisher's precise test was employed for statistical analysis of the histology data in the four groups.

Results

Evaluation of Fluoresce in Angiography

The frequency of occurrence for the various angiogenic responses (Grades 0, 1, 2, and 3) seen by fluorescein angiography for each cohort (treatment and control) for Groups 1 and 2 were compared using Mann Whitney U analysis. In group 1 (topical 0.3% FK-506), there was a significant difference between its controls at 5 weeks after retinal photocoagulation (p=0.05). In group 2 (topical 0.5% FK-506) there was a significant difference when compared to control eyes at 4 weeks and 5 weeks (P=0.018, P=0.021) (Table 1).

Histological Analysis of CNV

The four groups' fluorescence in angiography was evaluated using a Mann-Whitney U analysis, and the four groups' histology data were statistically analyzed using a Fisher's precise test.

Table 1. Chorioretinal responses in different weeks of management

	1st week	2nd week	3rd week	4th week	5th week
Control Group	No difference	No difference	No difference	No difference	No difference
Group 1 topical 0.3% FK-506	No difference	No difference	No difference	No difference	Significant difference p=0.05
Group 2 topical 0.5% FK-506	No difference	No difference	No difference	Significant difference p=0.018	Significant difference p=0.021

Discussion

Neovascular ARMD, is one of the five top causes of blindness in the USA and accounts for approximately 80% of cases with severe central vision loss [32,33]. The number of patients suffering from age-related macular degeneration is currently 196 million worldwide, while in 2040 this number is expected to be 288 million.4 Although many various therapies have been devised, unfortunately, there has still no satisfactory clinical therapy for the preservation of visual functions in patients with AMD.

The most appropriate animal model of CNV employs rats in which retinal lesions are done with an argon laser [34,35]. Although the model more likely imitates traumatic CNV, CNV resulting from ARMD and trauma the essential course (the dispersion of the basement membrane, migration and proliferation of vascular endothelial cells, and tubular formation) is analogous and often used for the assessment of the impact of new drugs to manage CNV [36].

Orally administered or sub-tenon injections of corticosteroids have been adopted for diagnosis of CNVMs provoked by the ocular histoplasmosis syndrome [37]. Intravitreally administered triamcinolone acetonide was incorporated into the treatment protocol during an uncontrolled trial test of CNVM management. Although interferon alpha has shown several angiostatic efficacies in vitro and animal models, human surveys have proved disheartening clinical performance and a Thalidomide, a potential anti-angiostatic drug, prevents post-receptor signal transduction for endothelial cell activation by signaling pathways. Prospective experimental and clinical studies with new drugs in people with exudative AMD are still ongoing.

Tacrolimus (FK506) C44H69NO12 · H2O is a natural product of soil microorganisms that have antifungal activity. Moreover it has similar pharmacological properties but even in concentrations 40–200 times lower than cyclosporine A. It has been widely used to handle graft rejection and graft-versus-host disease in various transplant recipients [17]. Tacrolimus enters in cell and affects intracellular immunophilin receptors FKBP12. The target of the FKBP12-FK506 complexes in T cells is calcineurin, a Ca2+-calmodulin-regulated serine/threonine-specific protein phosphate [38]. The activation of Calcineurin, which requires both Ca2+ and calmodulin binding, is acknowledged to mediate in T-cell initiation by dephosphorylation of the NF-AT translation factor. The functions of calcineurin in mammalian cells enclose roles in apoptosis and neutrophil migration [39]. Moreover, tacrolimus has been noted to depress the immune system in a calcineurin-independent way [40]. It appears to be related to an upregulation of transforming growth factor beta (TGF-B). This cytokine has not only remarkable immunosuppressive features but also initiates the deposition of matrix proteins and the development of tissue fibrosis [41].

According to Pleyer et al., the highest concentrations of FK506 after topical application of tacrolimus were discovered in the cornea and conjunctiva (200–1200 ng/g), with substantial amounts of the drug also manifest in the sclera throughout [26]. Lower concentrations were measured in the aqueous and vitreous humor (0.2–1.0 ng/g) in the oil-dissolved group (OD-FK506). All ocular tissues had high drug concentrations, but the LIP-FK506 group had the highest levels in the aqueous humor (5-28 ng/g) and vitreous humor (12-22 ng/g). Whitcup SM et al., used topically high-dose oil-dissolved FK506 and low-dose liposome-bound FK506 for the management of endotoxin-induced uveitis (EIU) and detected both were effective in inhibiting EIU. Lichlen et al. compared intravitreal Adalimumab, intravitreal Bevacizumab, and topical ESBA 105 molecule in terms of treatment in the experimental mouse CNV model and stated that the penetration of ESBA 105 molecule into the vitreous was good [34]. During the active period of the inflammation, the CNV grows to a particular size. Matrix metalloproteases (MMPs) are now produced by the vascular endothelium and macrophages, allowing the CNV to

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invade through tissue planes [42]. Tacrolimus encourages the deposition of matrix proteins like Macugen and RhuFABV at the beginning of the active stage after the initiation of the CNV and also stimulates the up-regulation of transforming growth factor beta (TGF-B) and TIMP3, which appears to limit the extent of the CNV, therefore the balance shifts toward antiangiogenic, anti-proteolytic, and anti-migratory activity resulting in the inflammatory inactive or involution stage of CNV [43].

Takahashi et al showed that topical nepafenac, as potent cyclooxygenase (COX-1) and (COX-2) inhibited CNV in three murine models and speculated that NSAIDs, exert their effect by decreasing macrophage-derived PGs [44]. It can be postulated that tacrolimus, like NSAIDs, presumably suppresses the inflammatory active faze of CNV like anecortave acetate, and triamcinalone acetonide.

As it is known, the best drugs in CNV treatment today are anti-VEGF drugs such as bevacizumab, ranibizumab, and aflibercept. However, the routes of administration of these drugs are intravitreal. To avoid possible side effects after intravitreal application in CNV treatment and in cases where the intravitreal application is contraindicated, various experimental drugs applied topically have been found. One of these drugs is pazopanib, a tyrosine kinase receptor antagonist. It also effectively inhibits VEGF receptors 1,2,3, PDGF, and FGF receptors. Therefore, topical use is effective in the treatment of CNV [45]. In another experimental CNV-induced mouse model, tetracycline has also been shown to inhibit angiogenesis after administration and to be effective in treatment. (Mechanism of fibrosis inhibition in laser-induced choroidal neovascularization by doxycycline) However, it should not be forgotten that tetracycline also has systemic side effects. Anti-angiogenic effects of the receptor tyrosine kinase inhibitor, pazopanib, on choroidal neovascularization in rats [45]. In another experimental CNV-induced rat model, topical calreticulin (known as vasostatin) has been shown to inhibit choroidal neovascularization [35]. We also found that treatment after topical tacrolimus application was effective in the experimental CNV model.

The impact of tacrolimus in rats as observed by FA and histopathological examination was handled. In the rat CNV model, angiogenesis attained its climax circa 2nd week and commence to drop, gradually going back to ordinary. Accordingly, groups 1 and 2 were applied topically tacrolimus following the first laser burn. The outcomes of the analogy of fibrovascular proliferation in eyes with CNV versus control eyes in groups 1 and 2 were statistically meaningful, with the best results achieved in-group 2 at the top of the suppression/inhibition of neovascularization in this design. Thus, our findings support that tacrolimus possesses significant anti-angiogenic characteristics in this rat design.

Conclusion

In conclusion, topical administration to the cornea is advantageous to deliver drugs to the eye, avoiding systemic side effects. Regarding previous studies, it has been shown that tacrolimus is a well-penetrating drug through the cornea, anterior, and posterior sclera, moreover following topical administration is able to reach effective concentrations, depending on application way and dose. In our study, there was a dose-response of topical FK-506 on the regression/inhibition of CNV. To our knowledge, this is the first study that tacrolimus applied topically to the cornea to suppress CNV. It suggests that topical application and delivery systems should be considered for tacrolimus about inhibition of CNV. This study needs to be performed in other experimental models and then clinical trials.

Conflict of interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical approval
Consent of Ethics was acquired from University of Tulane by Gholam A Peyman, MD Professor Emeritus.

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