

Inhibition of Choroidal Neovascularization by Intravitreal Ketorolac

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Objective: To determine the inhibitory effect of intravitreal nonsteroidal anti-inflammatory drugs on choroidal neovascularization (CNV) in an animal model of age-related macular degeneration.

Methods: Six laser burns of sufficient power to rupture the Bruch membrane were induced in the peripapillary area of each eye of 18 adult Brown Norway rats. Both eyes of each animal received the same 5- μ L intravitreal injection of 30 mg/mL of ketorolac tromethamine, 40 mg/mL of triamcinolone acetonide, or balanced salt solution. Fluorescein angiography was performed on days 14 and 21 after injection, animals were euthanized, and retinal pigment epithelium-choroid-sclera (choroidal) flat mounts were prepared. Areas of abnormal vascular leakage on fluorescein angiography and vascular budding on choroidal mounts were measured and quantified using an image analysis program.

Results: Intravitreal ketorolac significantly reduced CNV leakage on fluorescein angiography at 2 ($P < .001$) and

3 ($P = .006$) weeks compared with eyes injected with balanced salt solution, but intravitreal triamcinolone was a more potent inhibitor of CNV leakage than ketorolac ($P < .001$). Vascular budding on choroidal mounts was almost entirely suppressed with triamcinolone ($P < .001$) and significantly inhibited with ketorolac ($P = .009$).

Conclusion: Intravitreal ketorolac significantly reduced laser-induced CNV leakage and vascular budding as determined by fluorescein angiography and choroidal flat mounts, respectively, although this effect was less than that of triamcinolone.

Clinical Relevance: Intravitreal nonsteroidal anti-inflammatory drugs may be useful in the treatment of CNV owing to age-related macular degeneration or other causes and offer distinct clinical advantages over corticosteroids because of their lack of association with cataract formation or glaucoma.

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CHOROIDAL NEOVASCULARIZATION (CNV) is the most common cause of severe vision loss in patients with age-related macular degeneration (AMD) and contributes to substantial vision loss in younger patients with posterior uveitis, pathological myopia, and ocular histoplasmosis. Although the pathogenesis of CNV is complex and multifactorial, a growing body of scientific evidence indicates that inflammation plays a central role.¹⁻³ In support of this, intraocular corticosteroids have shown anatomical and visual benefit in patients with CNV.⁴⁻⁷ However, their routine use is limited by their adverse effects; thus, safer alternative anti-inflammatory agents would be beneficial.

Cyclooxygenase (COX) is an important enzyme in the inflammatory process and catalyzes the biosynthesis of prostaglandins. It can be detected in human choroidal neovascular membranes and is up-regulated in the retinal pigment epithelium (RPE) in response to proinflammatory cytokines.^{8,9} Prostaglandins amplify many other soluble mediators, including vascu-

lar endothelial growth factor (VEGF), a principal initiator of CNV.¹⁰⁻¹² In a variety of experimental systems, COX inhibition suppresses CNV development.¹³⁻¹⁵

Nonsteroidal anti-inflammatory drugs (NSAIDs) are potent inhibitors of COX enzymes and thereby the synthesis of prostaglandins. These drugs reduce postoperative inflammation and macular edema after cataract surgery and have antiproliferative and antiangiogenic effects.¹⁶⁻¹⁸ Topical or oral administration, however, does not provide high levels of drug to the retina; thus, intraocular administration may be a more effective means of treating posterior segment disease.^{16,19} Most important, NSAIDs do not cause cataracts or glaucoma (well-known and serious adverse effects of corticosteroids) and may offer a safer alternative.^{20,21}

Previous publications^{22,23} have reported that intravitreal ketorolac tromethamine is safe, results in a high concentration of drug in the retina, and markedly inhibits inflammation. We report herein the findings of a study designed to assess the intravitreal efficacy of ketorolac in a well-established laser-induced animal model of CNV.

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METHODS

ANIMALS

The Vanderbilt University Animal Care and Use Committee approved this investigation, and this study adhered to the Association for Research in Vision and Ophthalmology's statement on the use of animals in research. Eighteen adult, male Brown Norway rats (Charles River Laboratories Inc, Wilmington, Massachusetts) were selected for this study and maintained in a controlled environment with a 12-hour on/off light cycle. Food was available ad libitum. For all procedures, animals received an intramuscular injection of ketamine hydrochloride, 50 mg/kg, and xylazine hydrochloride, 5 mg/kg, for anesthesia. Pupils were dilated with topical tropicamide (0.5%), phenylephrine hydrochloride (2.5%), and atropine sulfate (1%). Proparacaine hydrochloride (0.5%) was applied for corneal anesthesia.

EXPERIMENTAL PROCEDURE

Ketorolac (Sigma-Aldrich Corporation, St Louis, Missouri) was acquired in powder form and dissolved in balanced salt solution (BSS; Alcon Laboratories, Fort Worth, Texas) to a final concentration of 30 mg/mL under sterile conditions and processed through a 22- μ m filter (Millipore Corporation, Bedford, Massachusetts). The concentration of ketorolac used in this study was previously reported to be nontoxic.²² Commercially available triamcinolone acetonide, 40 mg/mL (Kenalog-40; Bristol-Myers Squibb, Princeton, New Jersey), and BSS were used for the positive and negative control groups, respectively.

Laser photocoagulation was performed using a method similar to that described by Edelman and Castro.²⁴ In brief, on day 0, animals were positioned before a slitlamp (Carl Zeiss Meditec Inc, Jena, Germany) laser administration system. The fundus was visualized using a microscope slide coverslip with 2.5% hydroxypropyl methylcellulose solution as an optical coupling agent. An argon green laser (Coherent Inc, Santa Clara, California) was used for photocoagulation (532-nm wavelength, 360-mW power, 0.07-second duration, and 50- μ m spot size). This setting most reliably produced acute vapor bubbles indicative of the rupture of the Bruch membrane. In each eye, 6 focal laser photocoagulation lesions were concentrically induced approximately 2 optic discs from the center while avoiding major blood vessels. Eighteen animals were assigned to 1 of 3 experimental groups: ketorolac, triamcinolone, or BSS.

Before and immediately after injection, 0.5% moxifloxacin hydrochloride ophthalmic solution (Alcon Laboratories) was applied to the ocular surface. Intravitreal injections were performed immediately after the laser procedure. A single 5- μ L volume of drug (ketorolac or triamcinolone) or BSS was injected into the vitreous cavity under direct observation using a custom-made, beveled, 32-gauge Hamilton microinjector (Hamilton Company, Reno, Nevada). Both eyes of the same animal received the same material to avoid any potential crossover effect, and the final drug dosages of ketorolac and triamcinolone were 150 μ g and 200 μ g, respectively. Careful examination was performed after injection using a slitlamp, and no animals had traumatic cataract or vitreous hemorrhage.

ASSESSMENT

All animals underwent fluorescein angiography (FA) on days 14 and 21. For FA evaluations, 0.3 mL of 10% sodium fluorescein was administered intraperitoneally, and photographs of the right and left eye were taken at predesignated times (30 seconds and 1, 2, 5, 10, and 15 minutes). Ten-minute (1 minute within) phase angiograms (SD, 1 minute) were optimal and most

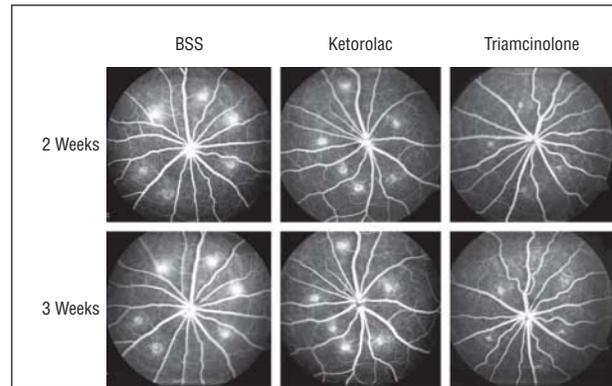


Figure 1. Representative fluorescein angiogram images of laser-induced choroidal neovascularization at 2 and 3 weeks after treatment with balanced salt solution (BSS), ketorolac, or triamcinolone.

consistent with early- to middle-phase angiograms and were used for all comparative analysis.

On day 21 after FA, animals were euthanized by cervical dislocation and eyes were enucleated and stored in 10% formalin for 2 hours. The RPE-choroid-sclera (choroidal) flat mounts were prepared as previously described.²⁵ In brief, the cornea and lens were removed, and after dissecting the retina from the eyecup and discarding it, radial cuts were made in all 4 quadrants to flatten the remaining tissue. The flattened RPE-choroid-sclera tissue was then mounted in gel mount (Biomediq Pty Ltd, Victoria, Australia). Endothelial cells were identified using fluorescein isothiocyanate-conjugated *Bandeiraea simplicifolia* isolectin B₄ (Sigma-Aldrich Corporation), and the elastin of the surrounding extracellular matrix was stained using goat antielastin antibody conjugated to Cy3 (Santa Cruz Biotechnology Inc, Santa Cruz, California). Choroidal flat mounts were visualized using the $\times 10$ objective lens of an epifluorescent compound microscope fitted with the appropriate excitation and emission filters (Provis AX-70; Olympus Corporation, Tokyo, Japan). Images of the neovascular lesions were captured using a digital camera attached to the Provis system (DP71; Olympus Corporation) coupled to a computer with image capture software (DP Controller; Olympus Corporation).

LESION MEASUREMENT

Choroidal neovascularization staining and leakage on FA and vascular budding on choroidal flat mounts were calculated by measuring the pixel area within the circumference of each rupture site as previously described.²⁶ In brief, digitized images were imported into Adobe Photoshop CS3 (Adobe Systems Inc, San Jose, California), and the CNV area was manually outlined by a masked retina specialist (S.J.K.) using the "quick selection tool."

STATISTICAL ANALYSIS

Results were expressed as mean (SD), and 95% confidence intervals were calculated. Comparison of mean values was performed using an unpaired *t* test with unequal variance. $P < .05$ was considered statistically significant.

RESULTS

There were 12 eyes (6 animals) per treatment group, and no eyes were excluded because of lens trauma, infection, or severe bleeding. Early- to middle-phase FA images at 2 and 3 weeks demonstrated consistent and visually detectable differences in rupture site staining and leakage among eyes injected with BSS, ketorolac, and triamcinolone (**Figure 1**).

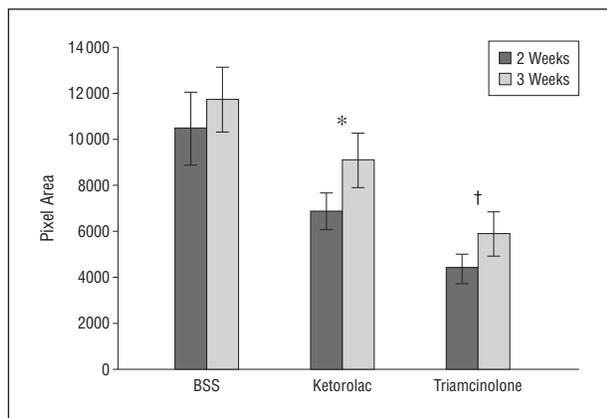


Figure 2. Mean pixel area of choroidal neovascularization lesions measured on fluorescein angiogram in eyes at 2 and 3 weeks after treatment with balanced salt solution (BSS), ketorolac, or triamcinolone. Error bars represent 95% confidence intervals. * Represents $P < .001$ and $P = .006$ at 2 and 3 weeks, respectively, compared with BSS; †, $P < .001$ at 2 and 3 weeks compared with BSS.

Intravitreal ketorolac significantly reduced CNV leakage on FA at 2 weeks ($P < .001$) when compared with eyes injected with BSS, and this effect was still significant at 3 weeks ($P = .006$; **Figure 2**). Intravitreal triamcinolone, however, was a more potent inhibitor of CNV leakage than ketorolac at 2 and 3 weeks ($P < .001$ for both).

All 3 groups showed an increase in rupture site leakage from 2 to 3 weeks, with the greatest absolute increase (2220-pixel area) occurring in ketorolac-treated eyes (**Figure 3**). Despite its continued presence in the vitreous, a statistically significant increase ($P = .01$) in CNV leakage (1482-pixel area) still occurred in triamcinolone-treated eyes at 3 weeks, suggesting that factors other than inflammation are involved in CNV progression.

Qualitative visual examination of choroidal flat mounts demonstrated almost complete inhibition of vascular budding by triamcinolone and markedly reduced vascular budding by ketorolac compared with BSS-treated eyes (**Figure 4**). Intravitreal ketorolac significantly reduced sprouting of vascular buds on choroidal flat mounts measured at 3 weeks compared with BSS-treated eyes ($P = .009$), but this inhibitory effect was less than that of triamcinolone ($P < .001$; **Figure 5**).

COMMENT

Previous publications^{22,23} have reported that intravitreal ketorolac is safe, provides a high concentration of drug to the retina, and markedly inhibits inflammation in a model of uveitis. To our knowledge, this is the first report on the efficacy of intravitreal ketorolac on CNV.

Choroidal neovascularization is the most common cause of severe vision loss in patients with AMD and results in substantial vision loss in several other conditions, including posterior uveitis, pathological myopia, and ocular histoplasmosis. Although the cause and pathogenesis of CNV are complex and remain incompletely understood, there is considerable evidence from human and animal studies that inflammatory and immunologic events play a central role.¹⁻³

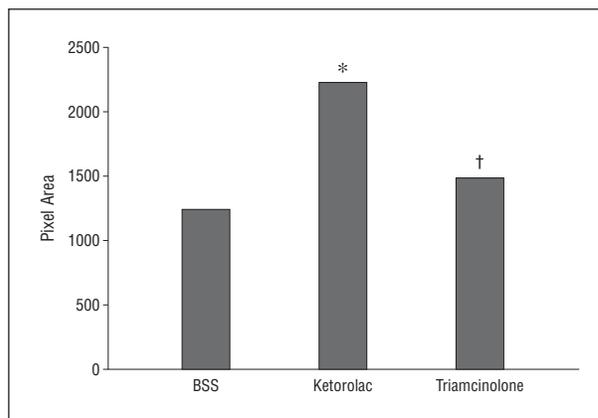


Figure 3. Mean increase in pixel area of choroidal neovascularization lesions from 2 to 3 weeks in eyes treated with balanced salt solution (BSS), ketorolac, and triamcinolone. * Represents $P = .003$ at 2 and 3 weeks; †, $P = .01$ at 2 and 3 weeks.

Macrophages are present in histologic specimens taken from patients with AMD and gather in areas of RPE atrophy and disruption of the Bruch membrane.^{2,27,28} Complement components (C3a, C5a, and C5b-9) are found in drusen, accumulate in laser-induced CNV complexes, and are capable of upregulating VEGF.^{25,29,30} The complement system comprises more than 30 soluble and membrane-bound proteins, which initiate, propagate, and regulate downstream inflammatory responses.^{31,32} The recent discovery that individuals homozygous for a variant of the gene for complement factor H (a negative regulator of the complement system) have more than a 7-fold increased risk of AMD strongly supports an inflammatory basis of CNV.^{33,34} In addition to AMD, impaired function of complement factor H is also associated with membranoproliferative glomerulonephritis type II.³⁵ This condition results from uncontrolled C3 deposition along basement membranes, and inhibition of COX reduces complement-induced glomerular epithelial cell injury.³⁶

COX is a rate-limiting enzyme in the inflammatory process and catalyzes the biosynthesis of prostaglandins. Two isoforms of COX, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in almost all tissues and is believed to be responsible for housekeeping functions. COX-2 expression is believed to be induced by a wide variety of pathological and physiologic stimuli. In the human retina, COX-1 can be detected in microglia, astrocytes, retinal ganglion cells, and amacrine cells, whereas COX-2 has been shown to be the predominant isoform in human RPE.³⁷ COX-2 can be detected in human choroidal neovascular membranes and is significantly upregulated in the RPE in response to proinflammatory cytokines.^{8,9}

Nonsteroidal anti-inflammatory drugs are potent inhibitors of COX enzymes and thereby the synthesis of prostaglandins. Prostaglandins have diverse physiologic and pathological effects within the eye. It is well established, for instance, that prostaglandins disrupt the blood-ocular barrier, increase vascular permeability, promote leukocyte migration, and stimulate ocular angiogenesis.^{16,17} In addition, prostaglandins induce VEGF, a central mediator of CNV, and inhibition of COX by NSAIDs prevents VEGF expression.^{10,38} Aspirin use may

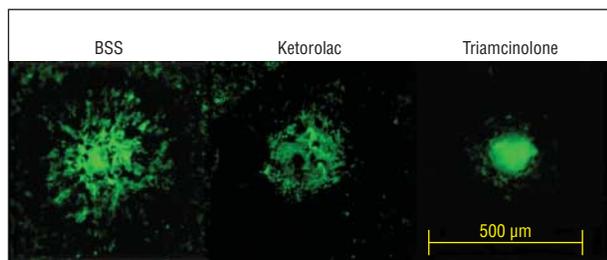


Figure 4. Representative choroidal flat mounts demonstrating laser-induced choroidal neovascularization and degree of vascular budding in eyes at 3 weeks after treatment with intravitreal balanced salt solution (BSS), ketorolac, or triamcinolone.

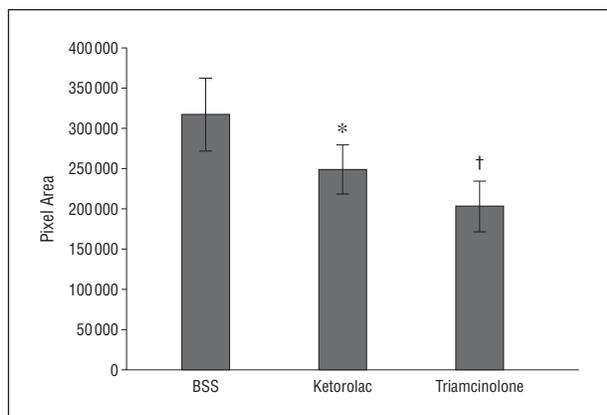


Figure 5. Mean pixel area of choroidal neovascularization lesions measured on choroidal flat mounts in eyes at 3 weeks after treatment with balanced salt solution (BSS), ketorolac, or triamcinolone. Error bars represent 95% confidence intervals. * Represents $P = .009$ compared with BSS; †, $P < .001$ compared with BSS.

be associated with a reduced risk of neovascular AMD, and topical and oral NSAIDs inhibit CNV in animal models.^{13,14,39}

Oral or topical administration of NSAIDs, however, provides limited drug administration to the retina.^{16,19,38} It has previously been reported that intravitreal injection of 3 mg of ketorolac results in vitreous and retinal concentrations approximately 1000-fold higher than what can be achieved via topical or oral administration, and despite its short half-life (4 hours), ketorolac significantly inhibited CNV leakage on FA and vascular budding on choroidal flat mounts in this study.²³ Although ketorolac's effect was significantly less than that of triamcinolone, triamcinolone has a much longer half-life and was clearly visible at 3 weeks in the vitreous of animals. A greater increase in CNV lesion size was observed between 2 and 3 weeks in eyes injected with ketorolac, suggesting that acceleration of CNV growth occurred in the drug's absence. In contrast, continuous topical (4 times daily) application of ketorolac provided sustained inhibition of CNV from 2 to 3 weeks (data not shown) and suggests that longer-lasting formulations of intravitreal ketorolac may have greater efficacy.

Ranibizumab (Lucentis; Genentech Inc, South San Francisco, California) is a humanized anti-VEGF antibody fragment that inhibits VEGF activity and is a remarkable advance in the treatment of CNV owing to AMD. Nevertheless, it is clear that no single therapy addresses the mul-

tifactorial pathogenesis of this disease. Furthermore, treatment with ranibizumab alone is not always effective, requires continuous monthly injections, and does not slow the underlying choroidal vascular atrophy with associated inflammation that is the presumed cause of dry AMD progression.⁴⁰ For these reasons and others, combination treatment with an anti-inflammatory agent has gained growing popularity and acceptance.^{7,41,42}

Laser-induced rupture of the Bruch membrane is the most frequently studied model for neovascular AMD and lends itself well to testing the inhibitory effects of intraocular drugs.⁴³ Although there is continued debate regarding the validity of extrapolating results obtained from laser-induced rupture of the Bruch membrane to that of degenerative changes occurring during decades, the underlying mechanism of CNV in these cases remains similar: neovascularization from the choroid occurs in areas of RPE atrophy and disruption of the Bruch membrane. Although a senescent AMD mouse model has recently been introduced, CNV occurs in a relatively small percentage of eyes and, at random times and locations, makes this model ill suited for intraocular drug studies.⁴⁴ Furthermore, in many conditions, such as choroidal rupture secondary to trauma, angioid streaks, ocular histoplasmosis, and posterior uveitis owing to multifocal choroiditis or punctate inner choroidopathy, the mechanism of CNV development may more closely resemble the laser-induced model.

In conclusion, intravitreal ketorolac significantly inhibited CNV leakage and vascular budding in an animal model of AMD, although the effect was less than that of triamcinolone. Nonsteroidal anti-inflammatory drugs, however, offer distinct clinical advantages over corticosteroids, but longer-lasting formulations may be necessary to achieve maximal effect.

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