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14. ABSTRACT. Greater than 10% of battlefield injuries occur to the eyes, resulting in significant morbidity. The potential for ocular infection by trauma is high, due to the types of organisms encountered in arid environments and the delay between injury and adequate treatment. This proposal was designed to analyze the effectiveness of antibiotics, anti-inflammatory drugs, and non-conventional agents targeting bacterial and host virulence factors, with the goal of improving the outcome of infections that would otherwise result in vision loss. The 2nd-year results highlight prompt and aggressive intravitreal therapy in preventing inflammation and vision loss. Delays in treatment result in vision loss, but may not result in loss of the eye, a cosmetic benefit. Our testing of additional anti-inflammatory agents with antibiotics did little to clarify whether these drugs are of any benefit during therapy. The use of vitrectomy to clear intraocular inflammation and damaged tissue may not be of much therapeutic benefit as well. With the first 2 years of study taken together, we have provided new information on improvements in treatment regimens that preserve vision and ocular architecture. Further analysis of non-conventional therapies will identify those that may be implemented for future treatment of blinding bacterial infections of the eye.					
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**Proposal PR064081 Department of Defense CDMRP
Improved Therapeutic Regimens for Treatment of Post-Traumatic Ocular Infections
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YEAR TWO ANNUAL PROGRESS REPORT

INTRODUCTION

Greater than 10% of the total number of battlefield injuries occurs to the eyes, resulting in significant morbidity.¹ The potential for ocular infection by penetrating injuries is significant, due to the nature of the organisms encountered in battlefield environments, and the delay between the time of injury and adequate therapeutic intervention. This proposal was designed to analyze the effectiveness of antibiotics, anti-inflammatory drugs, and non-conventional agents that target bacterial and host virulence factors, with the goal of improving the outcome of infections that typically result in significant vision loss. The goal is to sterilize the eye, arrest inflammation, and preserve vision following post-traumatic endophthalmitis.

BODY OF RESEARCH

Statement of Work (Abbreviated). Project tasks (experiments) included 7 experiments outlined in 2 specific aims that focused on testing conventional and non-conventional therapeutic regimens for the treatment of experimental post-traumatic *B. cereus* endophthalmitis. All experiments were performed using an experimental rabbit model of *Bacillus cereus* endophthalmitis. In the model, 100 bacilli/0.1 ml are injected into the mid-vitreous of one eye, while the contralateral eye is injected with 0.1 ml PBS (surgical control) or is left undisturbed (absolute control). At designated time points during infection, treatment is administered (intravitreal drug and/or surgery, with proper controls). At designated time points, eyes are electroretinographed (measurement of retinal function) and removed after euthanasia for analysis of myeloperoxidase activity (from inflammatory cells), drug concentrations, bacterial growth, and histology. The specific experiments in the Statement of Work are: 1) analysis of the efficacy of antibiotics or antibiotic combinations against experimental *Bacillus* endophthalmitis, 2) analysis of the efficacy of antibiotics against *B. cereus* spores in the eye before and after germination, 3) analysis of the efficacy of vitrectomy in conjunction with the most effective antibiotic for treatment of *B. cereus* endophthalmitis, 4) analysis of the efficacy of an anti-inflammatory agent, prednisolone acetate, in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis, 5) analysis of the efficacy of anti-TNF α in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis, 6) analysis of the efficacy of polyclonal antibody generated against *B. cereus* toxins (anti-toxin) in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis, and 7) analysis of the efficacy of polyclonal antibody generated against *B. cereus* flagella (anti-flagella) in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis.

All Figures can be found at the end of the text.

Experiment 1: Analysis of the efficacy of antibiotics or antibiotic combinations against experimental *Bacillus* endophthalmitis.

Summary: In Wiskur *et al.*², we published that intravitreal administration of antibiotics at 2 h or 4 h postinfection successfully preserved vision following experimental *B. cereus* endophthalmitis. These studies were expanded to analyze the fate of the eye after our latest analysis point of 8 h postinfection to determine whether delayed treatment prevented loss of the eye.

Experimental Design Summary: Eyes were intravitreally infected with 100 CFU *B. cereus*. 0.1 mL of gatifloxacin (0.3%), vancomycin (1.0%), or a combination of both antibiotics at these concentrations was intravitreally injected at 2, 4, or 6 h postinfection. Eyes were analyzed at 12, 24, and 36 or 48 h postinfection by bacterial quantitation, electroretinography, inflammatory cell quantitation, and histology (N \geq 5 eyes per group).

Progress To Date: All antibiotic treatments sterilized all eyes by 12 h postinfection. In terms of better retinal function outcome, 2 h treatment > 4 h treatment > 6 h treatment, and vancomycin = gatifloxacin > vancomycin + gatifloxacin. In terms of least inflammation after treatment, 2 h treatment > 4 h treatment > 6 h treatment, and vancomycin > gatifloxacin > vancomycin + gatifloxacin. The results demonstrate the importance of early intravitreal injection of either gatifloxacin or vancomycin (not both, as the retinal function retained was less and inflammation was greater with the combination than either antibiotic alone). In approximately half of cases of severe *B. cereus* endophthalmitis, the eye must be removed.^{3,4} Ocular architecture remained intact following 6 h treatment with either antibiotic, indicating that treatment delays of 6 h may not salvage vision, but may preserve the globe.

Data Not Shown: Bacterial counts (discussed in *Progress to Date*). See Experiment 4 for data of retinal function analysis, antibiotic concentrations, histology sections, and photographs of eyes from each treatment group at all endpoints.

Experiments Remaining: These experiments are complete. See Experiment 4 for further details.

Relevance: These results reiterate that early intravitreal treatment of posterior segment bacterial infections is critical for salvaging not only vision, but also the architecture of the globe itself. In the battlefield where treatment delays are expected, intravitreal administration of antibiotics following post-traumatic ocular injury may pre-empt infection and improve visual outcome.

Experiment 2: Analysis of the efficacy of antibiotics against *B. cereus* spores in the eye before and after germination.

Summary: During post-traumatic *Bacillus* endophthalmitis, spores likely enter the eye and germinate into viable bacteria that produce toxins and incite an explosive inflammatory response that is usually refractory to treatment. Our preliminary data demonstrates that spores can remain within the eye for up to 6 h and are not cleared. We propose to analyze the efficacy of antibiotics against *B. cereus* spores in the eye before and after germination.

Progress to Date: Spore preparations are being generated. *In vivo* experiments have not yet begun.

Experiment 3: Analysis of the efficacy of vitrectomy in conjunction with the most effective antibiotic for treatment of *B. cereus* endophthalmitis.

Summary: As noted in the experiments above, treatment at 6 h postinfection resulted in loss of retinal function and a severe degree of inflammation, but ocular architecture remained intact. In clinical cases of this severity, vitrectomy is used to remove posterior segment debris in order to potentially salvage vision. This study analyzed the effectiveness of vitrectomy used in conjunction with antibiotics for treatment of late-stage *B. cereus* endophthalmitis.

Experimental Design Summary: Rabbits were injected in the mid-vitreous with 100 CFU of vegetative *B. cereus*. Vitrectomy and concomitant intravitreal administration of vancomycin (1%) was performed at 4 h, 5 h, or 6 h postinfection. Controls include intravitreal vancomycin-treatment groups at the times stated above.

Progress To Date: Bausch & Lomb Inc. kindly provided a Millenium Transconjunctival Sutureless Vitrectomy (TSV25™) System at no cost for these studies. Two vitreoretinal specialists from the Dean A. McGee Eye Institute kindly volunteered their time and expertise and performed vitrectomies for this study. In terms of retinal function loss, vitrectomy offered little therapeutic benefit in conjunction with vancomycin treatment compared to previous studies analyzing vancomycin treatment alone at 6 h postinfection. Because a modest level of retinal function was retained in eyes undergoing vitrectomy + vancomycin at 4 h (Figure 1), we also tested this same regimen at 5 h postinfection to determine a treatment time when vitrectomy was of no benefit. The results at 5 h were similar to those seen with the 6 h vitrectomy + vancomycin regimen (Figure 1). For publication, these data must be compared to groups treated with vancomycin alone, but we expect similar results to those reported in Experiments 1 and 4. In the original proposal, we proposed to analyze treatment at time points after 6 h postinfection. These results suggest that those experiments would likely offer no evidence that vitrectomy is useful after 6 h postinfection, so those time points will not be analyzed.

Data Not Shown: Bacterial growth (discussed in *Progress to Date*), histology and photographs at 48 h endpoint.

Experiments Remaining: Vancomycin treatment without vitrectomy at 4 h, 5 h, and 6 h postinfection, quantitation of antibiotics and myeloperoxidase activities from tissues in above-mentioned vitrectomy experiments.

Relevance: These experiments demonstrate that vitrectomy may be of little use in salvaging vision once function is lost in this model. Its usefulness may lie in removal of intraocular inflammatory debris, thus removing potentially toxic host-derived products that may destroy tissue further.

Experiment 4: Analysis of the efficacy of an anti-inflammatory agent, prednisolone acetate, in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis.

Summary: In Wiskur *et al.*², we reported that intravitreal administration of dexamethasone (1.0%) with gatifloxacin (0.3%) or vancomycin (1.0%) did not limit inflammation or improve the outcome of infection compared with treatments with antibiotics alone. The present studies analyzed the effectiveness of prednisolone acetate in improving the outcome of experimental *B. cereus* endophthalmitis.

Experimental Design Summary: Eyes were intravitreally infected with 100 CFU *B. cereus*. 0.1 mL of vancomycin (1.0%), gatifloxacin (0.3%), or a combination of these antibiotics with prednisolone acetate (PredForte, Bausch & Lomb, 1.0%) was intravitreally injected at 2, 4, or 6 h postinfection. Eyes were analyzed at 12, 24, and 36 or 48 h postinfection by bacterial quantitation, electroretinography, inflammatory cell quantitation, and histology (N≥5 eyes per group).

Progress To Date: Vancomycin and gatifloxacin treatments sterilized all eyes. Eyes treated with prednisolone alone contained ~7.4 log₁₀ CFU/eye at 12 h, a quantity similar to that of untreated controls. Retinal function analysis, histology and photographs, myeloperoxidase activities, and intraocular antibiotic concentrations are summarized in Figures 2-5. Eyes treated at 2 h with either antibiotic maintained significantly higher retinal function compared to eyes treated with prednisolone alone (Figure 2). Overall, eyes treated at 6 h lost significant retinal function within 12 h (Figure 2), even though the eyes of all antibiotic-treated groups were sterile. Vancomycin treatment at 6 h resulted in less inflammation (Figures 3 and 4), less intraocular damage (Figure 4 histology), and less retinal function loss at 12 h (Figure 2) than did gatifloxacin treatment. Vancomycin-treated groups maintained concentrations greater than the MIC for *B. cereus*, but gatifloxacin concentrations were generally below the MIC at similar time points (Figure 5). The addition of prednisolone to vancomycin increased the intravitreal and aqueous humor concentrations of vancomycin (Figure 5), but did not reduce ocular inflammation when compared to antibiotic treatment alone (Figures 3 and 4). Early intravitreal injection of gatifloxacin or vancomycin improved the therapeutic outcome of *B. cereus* endophthalmitis. The addition of prednisolone to antibiotics did not result in a better visual outcome compared to those antibiotics used alone. Ocular architecture was preserved with intravitreal antibiotic treatment, even as late as 6 h postinfection. This study further demonstrates the critical importance of early therapeutic intervention in the treatment of *B. cereus* endophthalmitis.

Data Not Shown: Bacterial counts (discussed in *Progress to Date*) and clinical scoring of infection.

Experiments Remaining: These experiments are complete. See Reportable Outcomes for details.

Relevance: Loss of retinal function was similar in eyes treated with antibiotics with or without prednisolone. These results suggest that, like our recent findings of lack of benefit of antibiotics in conjunction with dexamethasone², the addition of prednisolone acetate to the therapeutic regimen does not improve the outcome of *B. cereus* endophthalmitis. The combination of vancomycin and gatifloxacin resulted in a more severe inflammation than that of those antibiotics used alone. Of note is the intact state of globes even after 6 h antibiotic treatment, suggesting that although retinal function is lost, globe architecture was intact following this therapeutic regimen.

Experiment 5: Analysis of the efficacy of anti-TNF α in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis.

Summary: We reported that TNF α is one of the pro-inflammatory cytokines synthesized in the eye during *B. cereus* endophthalmitis.¹⁰ Using transgenic mice deficient in TNF α , we identified this cytokine as being important in recruitment of inflammatory cells into the eye during *B. cereus* endophthalmitis.¹¹ Pilot experiments in mice demonstrated that anti-

TNF α reduced the inflammatory cell load when administered intravitreally alone at the time of infection, but the reduction was only 40% at 10 h postinfection. We proposed to analyze whether anti-TNF α combined with antibiotics effectively reduced inflammation and sterilized the eye during the early stages of *B. cereus* endophthalmitis.

Experimental Design Summary: Eyes were intravitreally infected with 100 CFU *B. cereus*. 0.1 mL of vancomycin (1.0%), a combination of vancomycin and anti-TNF α (Remicade, 0.5 ng), or anti-TNF α alone were intravitreally injected at 2, 4, or 6 h postinfection. Eyes were analyzed at 12 h postinfection by electroretinography and inflammatory cell quantitation (N \geq 3 eyes per group).

Progress To Date: Analysis of retinal function and inflammatory cell influx is presented in Figure 6. Overall, the combination of anti-TNF α + vancomycin did not improve B-wave function loss or extent of infiltrating inflammatory cells compared to vancomycin alone. To date, eyes have been analyzed at 12 h only. Treatment with anti-TNF α (Remicade) alone resulted in near complete retinal function loss and significant inflammation, as expected when antibiotics are absent.

Experiments Remaining: 1) Completion of these experiments with analysis of bacterial growth, histology and antibiotic concentrations 12 h, and analysis of all parameters at 24 and 36 h postinfection. N values must also be increased for some data points.

Relevance: Intravitreal administration of anti-TNF α (Remicade) to mouse eyes did not significantly reduce inflammation or alter function loss during *B. cereus* endophthalmitis. To date, no studies have analyzed the value of cytokine blockade in the treatment of intraocular infection, but studies do describe the use of anti-TNF α drugs in treating intraocular inflammation during uveitis.¹²⁻¹⁴ Our preliminary results demonstrate that TNF α blocking, even in the early stages of infection, may not improve the outcome of inflammation or infection. This is not surprising considering our findings of the production of additional chemotactic cytokines in the eye during experimental endophthalmitis in the mouse.¹¹ However, it is of interest to determine whether inflammation is kept at bay by anti-TNF α at a time past the 12 h time point, or if systemic or intraocular pre-loading of anti-TNF α will alter inflammation during infection.

Experiment 6: Analysis of the efficacy of polyclonal antibody generated against *B. cereus* toxins (anti-toxin) in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis.

Summary: We reported that toxin production in the eye during *B. cereus* endophthalmitis was responsible for the majority of retinal function loss during progressive infection.^{5,6} Others have reported that antisera raised to another ocular pathogen, *Staphylococcus aureus*, was effective in abrogating the effects of toxins during experimental endophthalmitis caused by that organism.^{7,8} We proposed a similar approach for treating *B. cereus* – combining anti-*B. cereus* toxin antisera with antibiotics during treatment at the early stages of infection when toxins are produced. The key is to kill the organism and arrest toxin activity in order to prevent toxin-induced retinal function loss.

Progress to date: Our efforts to raise polyclonal antibody to *B. cereus* toxins have been met with some difficulty. We are presently generating neutralized high-concentration toxin preparations to be sent to Rockland Inc. for custom polyclonal antibody synthesis. Once neutralizing antibody has been obtained, it can be used for administration with antibiotics during experimental *B. cereus* endophthalmitis. *In vivo* testing has not yet begun.

Experiment 7: Analysis of the efficacy of polyclonal antibody generated against *B. cereus* flagella (anti-flagella) in conjunction with the most effective antibiotic for *B. cereus* endophthalmitis.

Summary: We reported that *B. cereus* migration throughout the eye was important to the virulence of the organism.^{6,9} In an approach similar to that in Experiment 6, we proposed to combine anti-*B. cereus* flagella antisera with antibiotics during treatment at the early stages of infection when bacteria are moving within the posterior segment of the eye. The key is to prevent migration of the organism into niches where they may circumvent antibiotic action, while also killing the organisms.

Progress To Date: We are presently purifying *B. cereus* flagella for use in generating polyclonal antibody for administration with antibiotics during experimental *B. cereus* endophthalmitis. We may employ the same strategy as in Experiment 6 for generation of custom polyclonal antibody. *In vivo* testing has not yet begun.

KEY RESEARCH ACCOMPLISHMENTS

- Early and aggressive (i.e. intravitreal) antibiotic treatment of intraocular infection is critical in salvaging vision.
- Delayed treatment (i.e. at 6 h postinfection) does not prevent significant vision loss but does arrest deterioration of the globe itself.
- Addition of prednisolone acetate to vancomycin or gatifloxacin in the treatment of *B. cereus* endophthalmitis does not offer a therapeutic benefit over that of antibiotics used alone.
- Vitrectomy may offer a cosmetic, but not therapeutic benefit when employed in treatment of late-stage *B. cereus* endophthalmitis.
- Adjunct use of anti-TNF α may not alter the outcome of infection, but further studies are needed to determine whether inflammation is altered during infection or whether systemic or intraocular pre-treatment with anti-TNF α will alter inflammation.

REPORTABLE OUTCOMES

Experiments 1 and 4 are complete and were combined into abstracts accepted for presentation at the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting (May 2009) and the Military Health Research Forum (September 2009). These data were also submitted as a manuscript to *Antimicrobial Agents and Chemotherapy* (May 2009). An abstract with data from Experiment 3 will be submitted to the ARVO 2010 Annual Meeting (deadline December 2009).

CONCLUSIONS

Early intravitreal administration of antibiotics are key to preventing significant vision loss and loss of the eye itself following penetrating injury and potential intraocular infection. The importance of these studies lies in identifying the timing in which an eye can be salvaged, even though sight may be lost. On the battlefield, as well as during accidental trauma off the battlefield, the timing from injury to treatment is critical, as is the type of treatment administered. Numerous studies have demonstrated that for posterior segment infections, systemic and/or topical antibiotic treatment is relatively useless (reviewed in reference 15). We demonstrated that intravitreal administration of antibiotics (with or without anti-inflammatory drugs) salvages vision if given early and can rescue the globe if treatment is delayed. Although our work analyzes treatment of *B. cereus* endophthalmitis – the most devastating form of bacterial endophthalmitis – the results of these studies can be extrapolated toward treatment of infections with other vicious pathogens such as *Staphylococcus aureus* or *Streptococcus pneumoniae*, where courses of infection are slower but infection outcomes are just as devastating. The use of anti-inflammatory agents to alter inflammation remains controversial, and our results of prednisolone acetate/vancomycin or anti-TNF α /vancomycin administration do not help to clarify the issue. Future work targeted at virulence factor inhibition may aid in alleviating the issue of early retinal function loss despite antibiotic treatment.

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Figure 1. Efficacy of vitrectomy in conjunction with vancomycin for *B. cereus* endophthalmitis. Eyes were infected with *B. cereus* and treated with vitrectomy + vancomycin (1%) at various times postinfection. Control eyes were uninfected eyes treated with vitrectomy + vancomycin. Eyes were analyzed by electroretinography at 48 h postinfection or post-surgery. N≥3 eyes per group.

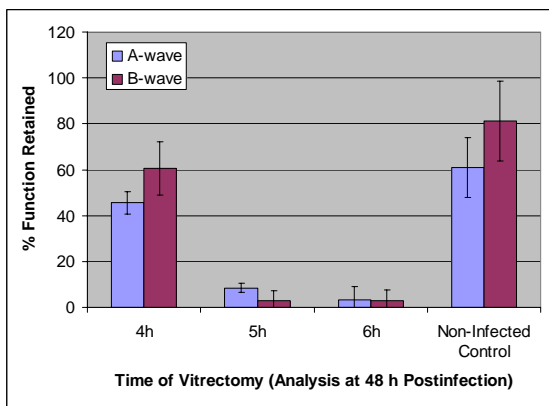


Figure 2. Retinal function analysis following antibiotic and/or prednisolone treatment of experimental *B. cereus* endophthalmitis. Scotopic ERGs were performed, and A- and B-wave amplitudes were recorded at 12, 24, and 48 h postinfection following treatment at 2 and 4 h postinfection or at 12, 24, and 36 h postinfection following treatment at 6 h postinfection. Reported values represent the mean±SEM of N=5 eyes per group (P≤0.05).

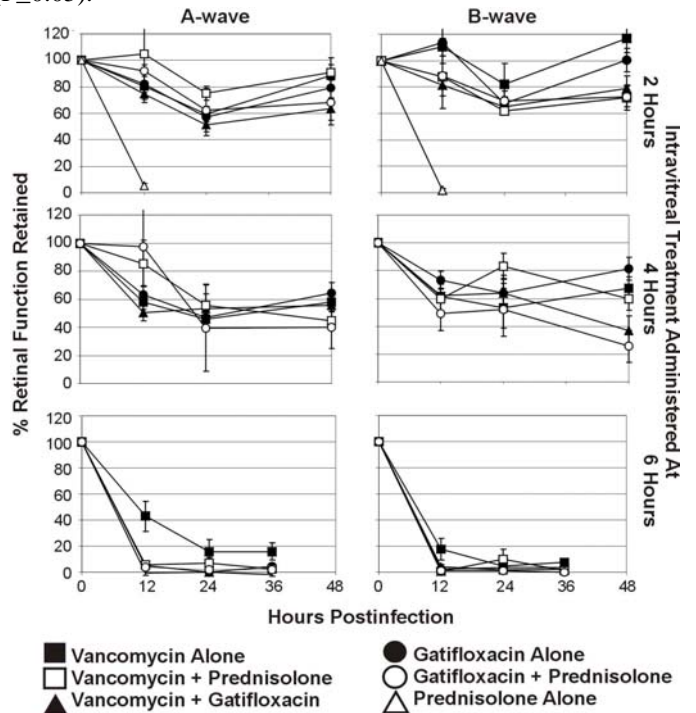


Figure 3. Myeloperoxidase activity following treatment of experimental *B. cereus* endophthalmitis. MPO activities of vitreous samples were quantified at 48 h postinfection for eyes treated and 2 and 4 h or at 36 h postinfection for eyes treated at 6 h postinfection. The values represent the mean \pm SEM of $N \geq 3$ eyes per group ($P \leq 0.05$).

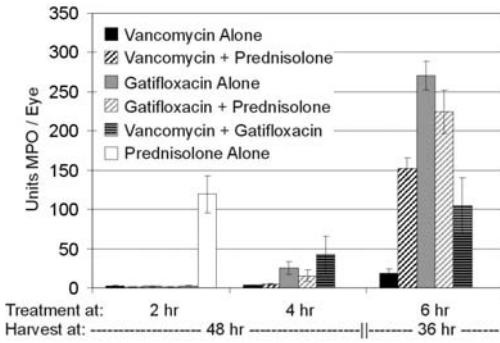


Figure 4. Histology and biomicroscopy following treatment of experimental *B. cereus* endophthalmitis. Eyes treated at 2 and 4 h postinfection were analyzed at 48 h postinfection, while eyes treated at 6 h postinfection were analyzed at 36 h postinfection. Eyes were photographed, enucleated and fixed in 10% formalin for 24 h, paraffin embedded, then sectioned and stained with hematoxylin and eosin by standard procedures. Eyes shown are representative of $N \geq 2$ eyes per group.

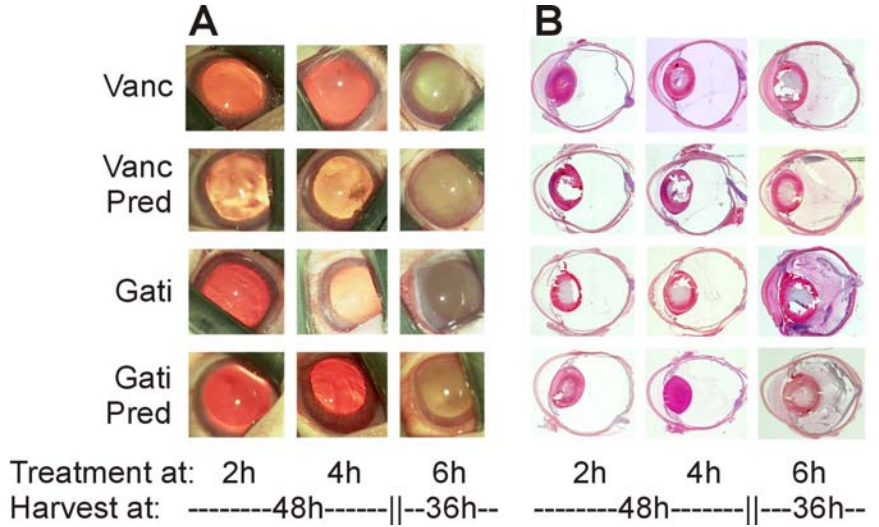


Figure 5. Intraocular antibiotic penetration following treatment of *B. cereus* endophthalmitis. Gatifloxacin and vancomycin concentration in the vitreous and aqueous chamber were quantified at 12, 24, and 48 h postinfection for eyes treated and 2 and 4 h, and at 12, 24, and 36 h postinfection for eyes treated at 6 h postinfection. The values represent the mean \pm SEM of $N \geq 4$ eyes per group ($P \leq 0.05$).

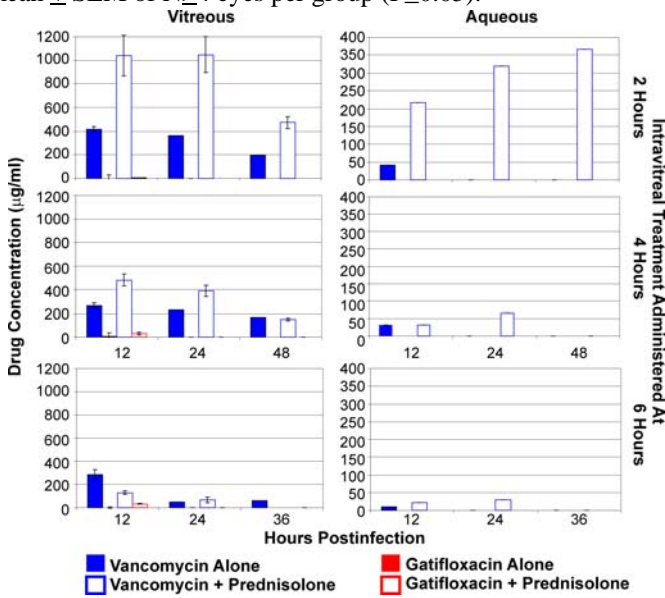


Figure 6. Retinal function and inflammatory cells in eyes with *B. cereus* endophthalmitis and treated with intravitreal vancomycin (Vanc), vancomycin + anti-TNF α (Vanc + Rem), or anti-TNF α alone (Rem) at 2, 4, or 6 h postinfection. Values are mean \pm SEM of $N \geq 3$ eyes/group.

