The anti-inflammatory potential of epigallocatechin gallate (EGCG) on tumor necrosis factor alpha and interleukin–10 expression in pseudomonas keratitis model (in vivo study on Rattus norvegicus rats model)

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ABSTRACT

Background: Pseudomonas aeruginosa infection is the primary cause of keratitis, leading to gradual corneal deterioration and resulting in scarring, thinning, and corneal perforation. Antibiotic treatment alone may prove ineffectual in certain instances, as the inflammatory process may remain despite the elimination of bacteria. Due to its antibacterial, anti-inflammatory, and antioxidant properties, Epigallocatechin gallate (EGCG) has the potential to be used as an adjuvant therapy for Pseudomonas keratitis. The objective of this study is to analyze the expression of TNF-α and IL-10 in a P. aeruginosa-induced keratitis model following the administration of moxifloxacin and EGCG eye drops.

Methods: Rattus norvegicus with keratitis induced by clinical isolate P. aeruginosa were randomly allocated to one of three treatment groups: the negative control group given NaCl 0.93% and benzalkonium chloride 0.01%; the group given moxifloxacin eye drops; and the group given moxifloxacin eye drops and EGCG 50 μg/mL on the third to fifth day after keratitis induction. Immunohistochemical staining of corneal tissue was utilized to analyze the levels of TNF-α and IL-10 expression.

Results: On the fifth day of subsequent keratitis induction, a statistically significant difference in TNF-α expression was observed across the groups (p=0.015). It was found between the moxifloxacin and EGCG 50 g/mL groups and the negative control group (p=0.008). IL-10 expression showed no significant difference across the groups (p=0.108).

Conclusion: TNF-α expression was significantly different among the three groups, whereas IL-10 expression was not significantly different.

Keywords: EGCG, Keratitis, Pseudomonas aeruginosa, TNF-α, IL-10.


INTRODUCTION

Keratitis refers to the inflammation of the cornea, which is marked by swelling of the cornea, inflammatory cell infiltration, and ciliary congestion. This condition can be caused by both infectious and noninfectious diseases. Among all occurrences of keratitis, bacterial keratitis comprises 90%, with Pseudomonas aeruginosa being the predominant pathogen detected in 23-50% of cases. Keratitis due to P. aeruginosa is marked by the formation of liquefactive necrotic lesions and an extensive accumulation of polymorphonuclear leukocytes (PMN), which can progressively damage the cornea and lead to cicatricial tissue formation, ultimately causing corneal perforation and permanent blindness, necessitating immediate treatment.1-4

Pseudomonas aeruginosa is the primary cause of bacterial keratitis, with a prevalence of approximately 21% in the United States and 24.7% in Indonesia. Data obtained from Dr. Cipto Mangunkusumo National Central Public Hospital (RSUPN Dr. Cipto Mangunkusumo or RSCM) in Jakarta and Dr. Soetomo General Academic Hospital (RSUD Dr. Soetomo) in Surabaya emphasize the substantial role of P. aeruginosa as a cause of keratitis. Drug-sensitive P. aeruginosa in India might result in corneal perforation in 12% of cases.5-8

The application of topical antibiotic therapy, in accordance with current standards, is most likely to lead to the remission of bacterial keratitis infection. However, the outcomes may still be unfavorable due to corneal melting, scarring, opacity, and perforation. Previous studies have suggested the necessity of corticosteroids and anti-collagenase as adjuvant therapy for bacterial keratitis. However, the utilization of corticosteroids remains controversial, and anti-collagenase application is based
on expert clinical experience, lacking clinical studies (randomized controlled trials) in humans.\(^8\)

Research on the pathogenesis of *P. aeruginosa* keratitis is ongoing to discover adjuvant therapies that can reduce the inflammatory response and prevent corneal clouding or perforation. *P. aeruginosa* virulence factors, including lipopolysaccharide (LPS) and flagellin, play a role in triggering inflammatory responses via the TLR4 and TLR5, MyD88, and NF-kB pathways. This process induces the production of TNF-α, which, through the RIPK1-RIPK3-MLKL pathway, can cause necroptosis of the cornea. The transcription factor NF-kB is also crucial in the polarization of M1 macrophages, which, under normal conditions, phagocytose a large number of pathogens but can exacerbate inflammation. M2 macrophages, with IL-10 production, assist in debris clearance, stimulate remodeling, and inhibit inflammation.\(^9-13\)

The anti-inflammatory, antibacterial, and antioxidant properties of polyphenolic compounds, including *Epigallocatechin gallate* (EGCG) found in tea leaves, have been extensively researched. Indonesia, being the primary global tea cultivator, offers significant potential in utilizing EGCG, a compound that has demonstrated efficacy in lowering inflammation and necroptosis in the eye. This includes conditions like dry eye disease and mice stroke models. The approach involves interfering with the NF-kB pathway, which results in a reduction in the synthesis of pro-inflammatory cytokines and TNF-α. Additionally, it inhibits the polarization of M1 macrophages and promotes the activation of M2 macrophages, resulting in an upregulation of IL-10 expression.\(^10\)

Based on the above description, the author conducted an *in vivo* experimental investigation using *Rattus norvegicus* as subjects that were treated with moxifloxacin and EGCG eye drops as adjuvant therapy for keratitis due to *P. aeruginosa* infection. The study evaluated the levels of TNF-α and IL-10 expression. It is expected that the results of this research will enable EGCG to serve as an adjuvant anti-inflammatory therapy, reducing inflammation following *P. aeruginosa* infection and, consequently, decreasing the rate of blindness due to extensive scar tissue formation on the cornea or corneal perforation.

**METHODS**

**Study design and setting**

This research constitutes an experimental study employing a randomized posttest-only design and was conducted at the Faculty of Veterinary Medicine, Universitas Airlangga, from September to October 2022.

**Experimental Animals**

The experimental animals used in this study were white Wistar rats. The study received ethical approval from the Ethics Commission for Basic and Clinical Science Research at the Faculty of Veterinary Medicine, Universitas Airlangga (No. 2.KEH.127.09.2022). Inclusion criteria encompassed white Wistar rats, male, aged 10–12 weeks, and weighing between 150–200 grams. The Faculty of Veterinary Medicine, Universitas Airlangga, conducted health assessments on the rats, ensuring a healthy condition and the absence of abnormalities in both eyes. Exclusion criteria comprised rats diagnosed with infectious diseases by a veterinarian and those exhibiting signs of endophthalmitis.

A total of 18 Wistar rats were induced for 48 hours by instilling a suspension containing laboratory isolates of *P. aeruginosa* (5 µl of *P. aeruginosa* suspension containing 2 x 10⁶ CFU) to become experimental rats for bacterial keratitis. Subsequently, the rats were randomly assigned to three groups. Treatment was then administered based on the group. Group K1, serving as the negative control, received eye drops with an EGCG solvent solution (NaCl 0.93% and benzalkonium chloride 0.01%) once every 4 hours. Group K2, the positive control, received 0.5% moxifloxacin eye drops once every 4 hours. Group K3 was administered moxifloxacin 0.5% eye drops and EGCG 50 µg/mL eye drops once every 4 hours. The administration of the different drugs in the K3 group was staggered by a 5-minute delay. On the third day, the corneal surface condition was examined, including the size of the corneal epithelial defect, using fluorescein tests. On the fifth day, a similar examination of the corneal surface was conducted, including the size of the corneal epithelial defect, using fluorescein tests. Subsequently, the experimental animals were terminated, and their corneal tissue was examined for the expression of TNF-α and IL-10 using immunohistochemistry following enucleation.

**Immunohistochemistry**

Immunohistochemical staining was performed on the surface tissues of the eyeball, including the conjunctiva and cornea, which had been enucleated on the fifth day. The H&E staining results were analyzed to assess the sufficiency of the paraffin blocks and the tissue condition for subsequent immunohistochemical testing. The paraffin blocks that were accessible underwent further histological examination using anti-TNF-α antibody (bs-2081R, biossusa.com) and IL-10 antibody (bs-0698R, biossusa.com).

The paraffin-blocked tissues were cut to a thickness of 3 µm, mounted on polylysine-coated glass slides, and incubated overnight at 45°C. The slides were deparaffinized, rinsed with running distilled water, then incubated with 3% H2O2 for 3 minutes before being rinsed again with running distilled water. The slides were treated with citrate buffer pH 6 at 95 °C for 45 minutes before cooling for 30 minutes and being washed twice with PBS for 3-5 minutes. The slides were then treated with blocking serum for 15 minutes and drained. The dry slides were treated with 1:500 diluted primary antibodies for 60 minutes before being washed twice with PBS for 3-5 minutes each. Next, secondary antibodies were incubated for 20 minutes. Following that, they were rinsed twice with PBS for 3-5 minutes. The slides were then incubated with HRV avidin for 10 minutes before being washed twice with PBS for 3-5 minutes each. DAB chromogen drops (1:50) were applied for 2 minutes, washed away with running water, and counterstained with Mayer’s hematoxylin, then rinsed with running water. The final stage was immersion in graded alcohol (70%, 96%, 100%) and xylene, followed by mounting.
Statistical Analysis

The data that was obtained was analyzed utilizing the SPSS version 26.0 program. The Shapiro-Wilk normality test was undertaken before the analysis. Clinical scores on the third day among the three groups were also analyzed with the Kruskal-Wallis test for sample homogeneity. The levels of TNF-α and IL-10 expression on the fifth day showed a non-normal distribution among the groups (p < 0.05). The analysis was conducted using the Kruskal-Wallis test, and if a notable disparity was seen, the Mann-Whitney test was later utilized. A p-value < 0.05 was considered to have statistical significance.

RESULTS

Description of Clinical Grades in Animal Models of P. aeruginosa-Induced Keratitis

On the third day after the induction of keratitis, the rats’ eyes were examined using a handheld slit lamp (Figure 1). It was observed that all samples met the inclusion criteria, leading to their random allocation into three groups. In this study, the lowest clinical score was grade 2, and the highest was grade 3. Clinical score grade 2 presented a manifestation of corneal inflammation resulting in dense opacity covering the pupil. Clinical score grade 3 depicted corneal inflammation leading to dense opacity throughout the cornea.

On the third day of clinical examination involving fluorescein tests, the mean clinical grade for all three groups was consistent at 2.17 ± 0.41. The absence of notable distinctions among the groups led to the conclusion that the clinical scores variable on the third day served as a confounding factor, with no significant variations among the three groups. This variable is considered ideal as a starting point.

Subsequently, all groups received treatment according to their respective assignments. The negative control group was topically administered NaCl 0.93% and benzalkonium chloride 0.01%, the positive control group was administered topical moxifloxacin 0.5%, and the treatment group was administered moxifloxacin 0.5% and EGCG 50 μg/mL. On the fifth day, another clinical examination was conducted involving infiltrates and fluorescein tests. The mean clinical grade in the negative control group increased from 2.17 ± 0.41 to 2.67 ± 0.52, marking the highest among the three groups. These results indicated that corneal opacities were more extensive and severe on the fifth day compared to the third day. The clinical grade in the positive control group was 2.50 ± 0.55, still lower than the negative control group. The mean clinical grade in the treatment group administered moxifloxacin 0.5% and EGCG 50 μg/mL was 2.33 ± 0.52, representing the lowest mean among the three groups (Table 1).

The clinical appearance of samples from the three groups on the third day and fifth day after induction is illustrated in Figure 2.

The Shapiro-Wilk normality test was conducted involving infiltrates and fluorescein tests. The mean clinical grade in the negative control group increased from 2.17 ± 0.41 to 2.67 ± 0.52, marking the highest among the three groups. These results were analyzed with the Kruskal-Wallis test for sample homogeneity. The levels of TNF-α and IL-10 expression on the fifth day showed a non-normal distribution among the groups (p < 0.05). The analysis was conducted using the Kruskal-Wallis test, and if a notable disparity was seen, the Mann-Whitney test was later utilized. A p-value < 0.05 was considered to have statistical significance.

Table 1. Average corneal clinical scores on the third and fifth days.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD Clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td></td>
</tr>
<tr>
<td>Negative control (NaCl 0.93% and benzalkonium chloride 0.01%)</td>
<td>2.17 ± 0.41</td>
</tr>
<tr>
<td>Positive control (Moxifloxacin 0.5%)</td>
<td>2.17 ± 0.41</td>
</tr>
<tr>
<td>Treatment Group (Moxifloxacin 0.5% and EGCG 50 μg/mL)</td>
<td>2.17 ± 0.41</td>
</tr>
</tbody>
</table>

The boxplot graph (Figure 3) displayed minimum and maximum values of 30 and 70, respectively, in the negative control group. The box’s size in the negative control group exhibited notable variability in comparison to the positive control group that received moxifloxacin, and the treatment group administered a combination of moxifloxacin and EGCG 50 μg/mL. The positive control group exhibited a range of values, with the lowest being 10 and the highest being 40. Similarly, the treatment group displayed a range of values, with the lowest being 10 and the highest being 30. In the positive control group, the extended whisker rod below the box suggested a leftward skewness (negative skewness) in the data.

Both the positive control group and the treatment group demonstrated decreased expression of the pro-inflammatory cytokine TNF-α in comparison to the negative control group. However, a notable disparity was only found in the treatment group when compared to the negative control group, but the difference in TNF-α expression between the negative control group and the positive control

Figure 1. Clinical features of the anterior segment of the rats’ right eye on the third day after keratitis induction. A. Anterior segment with a clinical score of grade 2 corneal inflammation, namely dense opacity on the cornea covering the pupil. B. Anterior segment with a clinical score of corneal inflammation grade 3, namely dense opacity throughout the cornea.

Figure 2. TNF-α Expression in the Cornea Tissue of Rat Models Induced by P. aeruginosa

TNF-α expression was assessed in the corneal tissue sections of the rats on the third day after keratitis induction in all groups. The results presented in the boxplot graph (Figure 3) displayed minimum and maximum values of 30 and 70, respectively, in the negative control group. The box’s size in the negative control group exhibited notable variability in comparison to the positive control group that received moxifloxacin, and the treatment group administered a combination of moxifloxacin and EGCG 50 μg/mL. The positive control group exhibited a range of values, with the lowest being 10 and the highest being 40. Similarly, the treatment group displayed a range of values, with the lowest being 10 and the highest being 30. In the positive control group, the extended whisker rod below the box suggested a leftward skewness (negative skewness) in the data.

Both the positive control group and the treatment group demonstrated decreased expression of the pro-inflammatory cytokine TNF-α in comparison to the negative control group. However, a notable disparity was only found in the treatment group when compared to the negative control group, but the difference in TNF-α expression between the negative control group and the positive control
group was not statistically significant. The considerable variability seen in the negative control group could potentially account for the absence of a statistically significant distinction between the negative control group and the positive control group.

The results of the immunohistochemical staining with TNF-α antibody on the corneal tissue of the rats on the fifth day after the induction of *Pseudomonas* keratitis revealed cells that appeared chromogenically brown and were positively immunoreactive (Figure 4). Differences were noted in the number of positively immunoreactive cells among the three groups. The negative control group displayed the highest count of positive immunoreactive cells, indicating a high expression of TNF-α in the corneal tissue. Subsequently, positive immunoreactive cells appeared to decrease in both the positive control group and the treatment group, respectively.

**IL-10 Expression in the Cornea Tissue of Rat Models Induced by *P. aeruginosa***

IL-10 expression in the corneal tissue sections of the rats was assessed on the fifth day after keratitis induction in all groups. The results presented in the boxplot graph (Figure 5) displayed minimum and maximum values of 20 and 70, respectively, in the negative control group. The box’s size in the negative control group showed substantial variation compared to the positive control group that received moxifloxacin, as well as the treatment group that received a combination of moxifloxacin and EGCG 50 μg/mL. The positive control group exhibited a range of values from 10 to 30, with 10 being the minimum and 30 being the maximum. Similarly, the treatment group showed a range of values from 20 to 40, with 20 being the minimum and 40 being the maximum. There was no discernible pattern in the expression of IL-10 in the three groups. The considerable variability seen in the negative control group may account for the absence of a statistically significant distinction between the negative control group and both the positive control group (K2) and the treatment group (K3). (*p < 0.05)

![Figure 2. Clinical features of samples in the three groups on the third day before treatment and the fifth day after treatment.](image1)

![Figure 3. Boxplot graph of TNF-α expression in each group. Statistical analysis demonstrated that the treatment group (K3) exhibited a notable decrease in TNF-α expression compared to the negative control (K1). There were no notable distinctions found between the negative control group (K1) and the positive control group (K2), as well as between the positive control group (K2) and the treatment group (K3). (*p < 0.05)](image2)

![Figure 4. Immunohistochemical staining with TNF-α antibody (bs-2081R, biossusa.com). A. TNF-α expression in the negative control group. B. TNF-α expression in the positive control group. C. TNF-α expression in the treatment group. Black arrows indicate positive immunoreactive cells expressing TNF-α. (400x Magnification)](image3)
corneal tissue of the rats on the fifth day after the induction of *Pseudomonas* keratitis revealed cells that appeared chromogenically brown and were positively immunoreactive (Figure 6). Variances were observed in the number of positive immunoreactive cells among the three groups. The negative control group exhibited the highest number of positive immunoreactive cells, indicating a high expression of IL-10 in the corneal tissue. Subsequently, the number of positive immunoreactive cells seemed to decrease in both the positive control group and the treatment group. Nevertheless, the treatment group exhibited slightly more positive immunoreactive cells in comparison to the positive control group.

**DISCUSSION**

*Pseudomonas aeruginosa* stands out as a frequent pathogen causing bacterial keratitis, leading to severe corneal damage. Although topical antibiotic therapy is generally efficacious, instances of poor prognosis arise due to corneal melting and opacification. This research has investigated the anti-inflammatory potential of EGCG in animal models of *Pseudomonas* keratitis. In this investigation, the authors focused on analyzing the levels of TNF-α and IL-10 expression following the topical administration of EGCG, combining it with moxifloxacin.

**Description of Clinical Grades in Animal Models of *P. aeruginosa*-Induced Keratitis**

In this study, clinical isolates of *P. aeruginosa* were employed to induce keratitis in experimental rats (*Rattus norvegicus*). The analysis of clinical scores on the third day revealed no significant differences among all samples. This observation identifies the clinical score on the third day as the optimal starting point for this study, with grades ranging from 2 to 3. Notably, none of the samples exhibited a mild clinical-grade following *P. aeruginosa* infection based on the clinical scores. This aligns with previous research by Thakur et al. (2002) and Kernacki et al. (2000), which demonstrated a moderate to severe clinical grade of keratitis in rats induced by *P. aeruginosa*.22,23

**TNF-α Expression in the Cornea Tissue of Rat Models Induced by *P. aeruginosa***

The analysis of TNF-α expression on the fifth day was based on previous research by Kernacki et al. (1998), indicating that TNF-α begins to decrease or approach normal baseline levels at 5-7 days.24 Significant differences in TNF-α expression were observed among the three groups, with the treatment group exhibiting the lowest percentage of TNF-α expression, followed by the positive control group and the negative control group. This emphasizes the anti-inflammatory role of EGCG as an adjuvant in the inflammatory process of *P. aeruginosa*-induced keratitis, particularly in preventing necroptosis.

Moxifloxacin, a lipophilic antibiotic with good water solubility, has demonstrated effectiveness against gram-negative bacterial ocular pathogens. Various studies have established the role of moxifloxacin in eliminating *P. aeruginosa*, thereby reducing the inflammatory process.24 In this study, a decrease in TNF-α expression in the treatment group in comparison to the negative control group was observed, indicating a potential to prevent sustained inflammation leading to necroptosis, corneal melting, and even corneal perforation. Multiple studies have emphasized the anti-inflammatory properties of EGCG, specifically in relation to the pro-inflammatory cytokine TNF-α and its involvement in necroptosis across various disease models. However, the previous studies did not investigate the effect of topical EGCG on TNF-α expression in the *Pseudomonas* keratitis model. Prior research indicated that...
EGCG effectively reduces the expression of TNF-α, the primary receptor for TNF-α. Furthermore, scientific evidence has demonstrated that EGCG effectively suppresses the levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-8, in the corneas of rabbits with dry eye disease. Additionally, topical administration of EGCG in animal models of Fusarium solani fungal keratitis resulted in decreased levels of TNF-α and IL-1β mRNA transcription on day 15 after treatment.

IL-10 Expression in the Cornea Tissue of Rat Models Induced by P. aeruginosa

This study also investigated the impact of moxifloxacin and EGCG eye drops as anti-inflammatory adjuvants on IL-10 expression in Pseudomonas keratitis. The results demonstrated the highest IL-10 expression in the K1 group, followed by K3, and the lowest in the K2 group.

The exact mechanism of IL-10’s role in the corneal inflammatory response remains unclear. IL-10 is typically absent in healthy corneas but is expressed prominently in CD11b+ cells, the primary corneal macrophages, during inflammation in the first and second weeks. This aligns with the findings of our study, where the highest IL-10 expression was detected in the negative control group (K1), aligning with increased TNF-α expression.

The combination of moxifloxacin and EGCG exhibited a non-significant increase in IL-10 expression compared to the treatment without EGCG. The impact of EGCG on IL-10 expression, serving as a marker for M2 macrophage polarization, is evident through its effect on macrophages. EGCG can elevate IL-10 transcription levels, thereby promoting M2 macrophage polarization while inhibiting LPS-induced M1 macrophage polarization. This modulation can help suppress inflammation and facilitate tissue repair.

Chen et al. (2023) conducted eye-related research in experimental animal models of Aspergillus fumigatus fungal keratitis, administering Atractylenolide (AT-I), an active compound isolated from the roots of the Atractylodes macrocephala plant. This compound, known for its role in innate immunity, exerts immunomodulatory effects on metabolic and immune functions, particularly on pro-inflammatory cytokines associated with the NF-kB pathway, similar to the mechanism of action of EGCG. Their study revealed a significant decrease in IL-10 expression in the fungal keratitis group treated with natamycin and AT-I. IL-10 acts to inhibit inflammatory protein responses, preventing bacterial and viral infections, as well as mitigating excessive tissue damage caused by pro-inflammatory responses. IL-10 also plays a crucial role in enhancing corneal transparency by reducing neutrophil and macrophage infiltration and suppressing inflammatory responses in the early stages of inflammation.

Several limitations exist in this research. Firstly, the absence of a group only receiving EGCG creates ambiguity regarding whether the disparity between the positive control group and the treatment group is a result of a synergistic effect involving the addition or potentiation of moxifloxacin and EGCG. Secondly, the measurement of IL-10 expression lacks specificity for keratocytes or macrophages, making it challenging to ascertain the anti-inflammatory effect of EGCG through a mechanism that promotes M2 macrophage polarization. Thirdly, this study solely investigated cytokine expression at a single point in time throughout the inflammatory phase, disregarding the dynamic nature of cytokine expression. Hence, it is imperative to consistently monitor the expression of TNF-α and IL-10 in order to accurately capture the dynamic changes in the inflammatory response.

CONCLUSION

The administration of EGCG and moxifloxacin resulted in a considerably reduced TNF-α expression, a cytokine known for its pro-inflammatory properties, compared to the other groups. EGCG shows potential as an anti-inflammatory adjuvant for Pseudomonas keratitis by interfering with the expression of TNF-α. Additional research is required to investigate the influence of EGCG on IL-10 expression and its potential for improving clinical outcomes in keratitis.

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AUTHORS’ CONTRIBUTION

All authors participated in the analysis of the data, the writing process, and the revision of the publication and collectively agreed to take responsibility for all elements of this work.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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ETHICAL CLEARANCE

The study has obtained ethical clearance from the Ethics Commission for Basic and Clinical Science Research at the Faculty of Veterinary Medicine, Universitas Airlangga with reference letter number 2.KEH.127.09.2022.

REFERENCES


