INTRODUCTION

Corneal opacities rank as the fourth most prevalent ocular health issue on a global scale. Bacterial keratitis is the primary etiological factor contributing to the development of corneal opacities, with approximately 50% of cases attributed to keratitis caused by Pseudomonas aeruginosa. Pseudomonas aeruginosa keratitis is frequently observed in 23-50% of instances of bacterial keratitis. This condition is distinguished by the presence of liquefactive necrotic lesions and a significant build-up of polymorphonuclear leukocytes (PMNs). These PMNs have the potential to progressively harm the cornea, resulting in various complications such as corneal clouding, corneal thinning, corneal perforation, and permanent blindness.1-5

In typical circumstances, the cornea is safeguarded by a set of defensive processes that serve to impede the infiltration of organisms into its underlying layers. The immunological state of a healthy cornea, which is constantly exposed to external pathogens, allergens, and pollutants, is characterized by a state of immunological quiescence, resulting in a minimal pro-inflammatory response. The overactivation of the proinflammatory response might lead to detrimental effects. In the ocular tissues of healthy and unimpaired rats, there is a demonstrated ability to withstand a range of approximately 10⁹ to 10¹¹ colony forming units (CFU/mL) of Pseudomonas aeruginosa and Staphylococcus bacteria. Staphylococcus epidermidis and Staphylococcus aureus, two significant corneal pathogens, do not induce adhesion, colonization, or penetration into the underlying layers.6-8

The administration of conventional topical antibiotics continues to be associated with adverse effects, such as corneal opacities and corneal perforation, due to the inflammatory response leading to the deterioration of adjacent healthy tissue. In order to mitigate this morbidity, the implementation of adjuvant therapy is necessary. The latest scientific investigation...
pertaining to epigallocatechin gallate derived from green tea has revealed its potential as an anti-inflammatory agent in the treatment of dry eye illness. This action is believed to be achieved through the suppression of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB). The role of NF-κB in initiating the production of IL-6 through TNF-α and the subsequent recruitment of neutrophils in the inflammatory response of Pseudomonas aeruginosa keratitis leads to cellular demise.9,11

Clinically, this process manifests as corneal turbidity, corneal thinning, and ultimately corneal perforation. The role of inflammatory severity markers, such as IL-6 and neutrophils, has been extensively documented in academic literature. Research has been initiated on the creation of IL-6 and neutrophils intervention. To date, there has been limited scholarly discourse on the topic of adjuvant therapy, specifically focusing on the use of EGCG, and its potential to enhance the morbidity outcomes associated with corneal opacities in cases of keratitis caused by Pseudomonas aeruginosa. The existing literature consists of a small number of studies and references.6-11

Neutrophils constitute the predominant population of leukocytes in the bloodstream, typically ranging from 4000 to 10,000 cells per microliter (µL). The production of neutrophils is induced by cytokines known as colony-stimulating factors (CSFs), which are released by various cell types in response to infection. These CSFs act on stem cells in the bone marrow, promoting the proliferation and maturation of precursor cells for neutrophils, including myeloblasts, pro-myelocytes, myelocytes, and metamyelocytes, ultimately leading to the development of mature neutrophils. Neutrophils are the primary cells that exhibit an initial response to various infections, particularly those caused by bacteria and fungi. Moreover, during both homeostasis and acute inflammation, neutrophils are the prevailing cell type. The process of phagocytosis, wherein neutrophils and monocytes in the bloodstream engulf and eliminate germs, is characterized by a series of rapid events including chemotaxis, phagocytosis, degranulation, respiratory burst, and the subsequent release of oxidants to effectively neutralize bacteria. During the inflammatory phase, neutrophils undergo an up-regulation of macrophages, T-cells, TNF-α, IL-8, IL-1, and IL-6.12,14

Chemokines present in keratitis caused by Pseudomonas aeruginosa, which occurs as a result of NF-κB activation, have a function in the recruitment of neutrophils to the infection site. Subsequently, these neutrophils engage in active phagocytosis and elimination of Pseudomonas aeruginosa. However, it is important to note that the degranulation of neutrophils, leading to the release of proteolytic enzymes and reactive oxygen species, can induce significant tissue damage. This tissue damage is a necessary component of the neutrophils’ role in limiting the spread of bacteria.13,15,16

Recent in vivo research has provided evidence indicating that neutrophils possess additional activities beyond their role in host immune defense such as combating infection through processes like phagocytosis, degranulation, and cytokine generation. Notably, these investigations have shown the formation of neutrophil extracellular traps (NETs) as another mechanism employed by neutrophils in the battle against infections. Based on recent study, the role of NETs has been identified to align with earlier research and theoretical frameworks, specifically in terms of its ability to decrease bacterial populations and induce corneal damage. Neutrophils represent a potential area of focus for therapeutic intervention and scientific advancement. Neutrophils were seen to emerge on the initial day and exhibited an increase on the third day, followed by a decline on the seventh day after infection in the negative control rats. Notably, the second day post-infection marked the commencement of therapy and combination therapy.11

IL-6 is a cytokine with proinflammatory properties that serves as a crucial mediator in both immune responses and inflammatory processes. It is primarily synthesized by leukocytes and exerts its effects on various leukocyte populations. Dendritic cells, mast cells, and macrophages are the primary sources of cytokines in the context of innate immunity. The type I cytokine family comprises many members, including but not limited to IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12A (p35), and several others. The primary origins of IL-6 are macrophages, endothelial cells, and T cells involved in adaptive immunity. Conversely, in the cornea, IL-6 is produced by corneal epithelial cells, corneal endothelium, macrophages, and keratocytes.13,15 IL-6 also has a regulatory function by inhibiting the production of interleukin-1 (IL-1) and tumor necrosis factor (TNF), so attenuating the inflammatory response and potentially mitigating ocular surface damage. Interleukin-6 (IL-6) serves as a regulator for the proliferation of epithelial cells and the adhesion between cells. Nevertheless, there is still a lack of clarity about the specific role of indigenous corneal cells and invading inflammatory cells in the up-regulation of IL-6.12,17

The primary polyphenol present in green tea (Camellia sinensis) is EGCG. EGCG has been widely recognized for its ability to exert anti-inflammatory and antioxidant properties on diverse cellular populations inside the human body, including the corneal cells. The antibacterial efficacy of EGCG against Pseudomonas aeruginosa has been investigated through multiple in vitro experiments, which have demonstrated notable outcomes at specific concentrations. Moreover, EGCG has been found to inhibit the production of Pseudomonas aeruginosa biofilms. Multiple in vivo studies have also shown evidence of the impact of EGCG on dry eye disease and its ability to decrease necroptosis in animal models of stroke in rats.6,7,9,15,18

METHODS

The research employs an experimental design featuring a post-test only control group. The research was conducted in September 2022 at the Central Laboratory of Stem Cell Research and Development and the Faculty of Veterinary Medicine, Universitas Airlangga, located in Surabaya, Indonesia. A total of 18 adult Wistar white rats (Rattus norvegicus) were included in the study through random allocation. The samples were allocated into two groups for the experiment: the test
group, which received a combination of moxifloxacin 0.5% and EGCG 50μg/mL, and the control group, which consisted of a negative control group that was given NaCl 0.93% and benzalkonium chloride 0.1% solution, as well as a moxifloxacin 0.5% group serving as a positive control. The topic of this study consisted of male Wistar white rats, aged between 3 and 4 months, with a body weight ranging from 150 to 200 grams. The eyes of the subject were thoroughly checked by the Faculty of Veterinary Medicine, Universitas Airlangga, and found to be in a state of good health with no detectable anomalies.

Rat that satisfied the predetermined criteria for inclusion were subjected to a corneal epithelial defect procedure, wherein three distinct locations in the central region of the cornea of the right eye were scratched using a technique resulting in a 1 mm long defect. Subsequently, these rats were induced by instilling a suspension containing 5μl of Pseudomonas aeruginosa at a concentration of 2x10⁶ colony-forming units. All groups were subjected to a 48-hour observation period during which no antibiotics or other medications were administered. Subsequently, an examination was conducted on the corneal surface, followed by a random allocation into three distinct groups: the negative control group (K1), the moxifloxacin 0.5% group (K2), and the combination EGCG group (K3).

The present investigation involved the examination of the ocular surface on three different days: the first day prior to the induction of Pseudomonas aeruginosa keratitis, as well as on the third and fifth days of the study, utilizing the fluorescein test. The fluorescence test involves the introduction of fluorescein dye into the eye, followed by irrigation with sterile distilled water. Subsequently, the corneal condition is assessed utilizing a slit lamp, also known as a biomicroscope, which employs blue light.

Following the process of induction, the participants were assigned to groups in a random manner. The administration of medication occurred on the third day, precisely 48 hours subsequent to the initiation of bacterial keratitis. The participants in the K1 group were administered a single drop of a solution containing a combination of NaCl 0.93% and benzalkonium chloride 0.1% at intervals of 4 hours. The K2 cohort received a single drop of moxifloxacin 0.5% at intervals of 4 hours. The K3 group received a regimen of combination moxifloxacin 0.5% and EGCG 50 μg/mL ocular drops administered at intervals of 4 hours. The administration of successive doses of K3 group medicines occurred at 5-minute intervals. On the third day, an examination of the surface of the eyeball was conducted, which involved assessing the dimensions of the corneal epithelial defect. On the fifth day, an examination was conducted to observe the surface of the cornea, specifically focusing on the measurement of the corneal epithelial defect. Subsequently, all experimental animals were euthanized, and enucleation procedures were performed to facilitate immunohistochemistry analysis of neutrophil count and IL-6 expression.

The rat eyes that had undergone enucleation were subjected to fixation in a 10% buffered formalin solution in order to inhibit autolysis and decomposition. This fixation process was carried out at room temperature for a duration of 24 hours. Following fixation, the eyes were embedded in paraffin for further analysis. The corneal tissue, which was kept separate from other tissues, was immersed in a solution of 10% neutral buffered formalin and allowed to fix for a duration of 48 hours at ambient temperature. The tissue specimen, measuring 20 mm × 30 mm with a thickness ranging from 2 to 4 mm, is treated with formalin at a ratio of 2 ml per 100 mg of tissue to ensure optimal fixation. The paraffin block underwent sectioning using a microtome with a thickness of 4μm, followed by staining using hematoxylin and eosin (HE) as well as immunohistochemistry techniques.

The analysis of neutrophil count and IL-6 expression was conducted using its percentage. The process involves the multiplication of the percentage score of cells or areas exhibiting immunoreactive positivity for the chromogen brown with the score representing the intensity of coloration observed on the cells. The data pertaining to each sample consists of the average of cells/expression percentage observed within five distinct fields of view, each magnified at 400x. The data was collected and subjected to analysis using the Kruskal-Wallis test, followed by a post hoc test due to the non-normal distribution of the data. The statistical significance of the results was determined by assessing whether the p-value was less than 0.05.

RESULTS

The evaluation of corneal inflammation is conducted by the utilization of a slit lamp, whereby clinical scores are assigned to measure the severity of inflammation both prior to and subsequent to exposure. Using the fluorescence test, it was determined that all patients exhibited a corneal defect, which was subsequently evaluated through the use of clinical scoring. The clinical scores of all individuals were recorded as 2 and 3. A clinical score of 2 signifies the presence of corneal inflammation, resulting in cloudiness of the cornea that may partially or fully obstruct the pupil. Conversely, a clinical score of 3 indicates corneal inflammation leading to complete cloudiness, encompassing the entire anterior segment (Figure 1 and Figure 2).

The mean clinical scores of K1, K2, and K3 were collected using fluorescein examination on the 3rd day, resulting in a mean score of 2.17 ± 0.41. On the 5th day, the mean clinical scores of K1, K2, and K3 were 2.67 ± 0.52, 2.33 ± 0.52, and 2.17 ± 0.41, as shown in Table 1. The findings of this study indicate a reduction in the clinical score magnitude following each treatment.

The results of the Kruskal-Wallis test indicate that there was no statistically significant difference in clinical scores among the K1, K2, and K3 groups on the 3rd day (p=1.00) and 5th day (p=0.269). It is important to note that statistical significance is typically defined as a p-value less than 0.05.

The neutrophil count and IL-6 expression involved determining the proportion of cells expressing neutrophils and IL-6 out of a total of 100 corneal cells surrounding the corneal defect. This assessment was conducted using a light microscope at a magnification of 400x. The findings from the immunohistochemical analysis using Hematoxylin-eosin staining, along with the application of
µg/mL EGCG can significantly reduce the number of neutrophils in Rattus Norvegicus rats with the Pseudomonas aeruginosa keratitis model compared to only being given solvent. The results of the Mann Whitney test between the 0.5% Moxifloxacin group and the 0.5% Moxifloxacin combination and EGCG 20 µg/mL group found that there was no significant difference between the two groups (p=0.140). These results indicate that the addition of 20 µg/mL EGCG was not significant in reducing the number of neutrophils in rat Rattus Norvegicus rats with the Pseudomonas aeruginosa keratitis model when compared to the group that was only given 0.5% moxifloxacin. However, the combination of 0.5% moxifloxacin and 20 µg/mL EGCG reduced the number of neutrophils in Rattus Norvegicus rats with the Pseudomonas aeruginosa keratitis model when compared to the group that was only given solvent. This also shows that in reducing the number of neutrophils in Rattus norvegicus rats with the Pseudomonas aeruginosa keratitis model, EGCG cannot be given without moxifloxacin 0.5%.

The data shown in Table 1 demonstrates that the mean percentage of IL-6 expression in the control group, which received the mixed NaCl solution, was 31.67±11.69. However, it is worth noting that the substantial standard deviation suggests a considerable degree of variability within the dataset. The mean values for the positive control group and the test group were 22.5±6.89 and 16.67±5.16, respectively. The findings of this study revealed that within the three treatment groups, the negative control group exhibited the highest percentage of IL-6 expression, whereas the combined EGCG group demonstrated the lowest proportion.

The results from the Kruskal-Wallis test (Figure 5) indicate a statistically significant variation in IL-6 expression among the three experimental groups, namely the negative control group (solvent), positive control group (0.5% Moxifloxacin), and the test group (0.5% Moxifloxacin and 20 EGCG µg/mL). The findings from the Kruskal-Wallis test (Figure 5) indicate a statistically significant variation in IL-6 expression among the three experimental groups, namely the negative control group (solvent), positive control group (0.5% Moxifloxacin), and the test group (0.5% Moxifloxacin and 20 EGCG µg/mL).

Figure 1. The clinical stages of three groups (K1, K2 and K3) before and after induction, 3rd and 5th day after treatment.

Figure 2. The clinical stages of three groups (K1, K2 and K3) 3rd and 5th day after treatment by fluorescein examination.

anti-neutrophils and anti-IL-6 antibodies, revealed a comparison of neutrophil count and IL-6 expression in the area affected by Pseudomonas aeruginosa keratitis across different groups (Figure 3). The cytoplasm of cells that exhibited neutrophils and IL-6 expression displayed a brown color.

A decrease in the mean count level of neutrophils was observed among the treatment groups K1, K2, and K3. The study demonstrated that the average percentage of neutrophils in the K3 group was significantly lower compared to the K2 group, with values of 38.33±14.72 and 49.17±12.01, respectively. The results of the Kruskal-Wallis test analysis (Figure 4) indicated a statistically significant difference among the K1, K2, and K3 groups (p<0.05).

The results of the Mann Whitney test (Figure 4) between the negative control group (solvent) and the combination group of Moxifloxacin 0.5% and EGCG 20 µg/mL found that there was a significant difference between the two groups (p=0.032). These results indicate that the addition of 20 µg/mL EGCG can significantly reduce the number of neutrophils in Rattus Norvegicus rats with the Pseudomonas aeruginosa keratitis model compared to only being given solvent. The results of the Mann Whitney test between the 0.5% Moxifloxacin group and the 0.5% Moxifloxacin combination and EGCG 20 µg/mL group found that there was no significant difference between the two groups (p=0.140). These results indicate that the addition of 20 µg/mL EGCG was not significant in reducing the number of neutrophils in rat Rattus Norvegicus rats with the Pseudomonas aeruginosa keratitis model when compared to the group that was only given 0.5% moxifloxacin.

However, the combination of 0.5% moxifloxacin and 20 µg/mL EGCG reduced the number of neutrophils in Rattus Norvegicus rats with the Pseudomonas aeruginosa keratitis model when compared to the group that was only given solvent. This also shows that in reducing the number of neutrophils in Rattus norvegicus rats with the Pseudomonas aeruginosa keratitis model, EGCG cannot be given without moxifloxacin 0.5%.
was further conducted using the Mann Whitney test. The statistical analysis using the Mann Whitney test (Figure 5) revealed a significant difference (p<0.05) between the negative control group (solvent) and the combination group of moxifloxacin 0.5% and EGCG 20 µg/mL. The findings of this study suggest that the administration of a 20 µg/mL EGCG combination leads to a notable decrease in IL-6 expression in Rattus Norvegicus rats with the Pseudomonas aeruginosa keratitis model, in comparison to the administration of solvent alone.

The study compared the test results of two groups: the 0.5% moxifloxacin group and the 0.5% moxifloxacin combination with EGCG 20 µg/mL group. The findings of the study indicate that there was no statistically significant difference seen between these two groups (p>0.05). The findings of this study suggest that the inclusion of a 20 µg/mL EGCG combination, administered 5 minutes after the injection of 0.5% moxifloxacin, did not yield a statistically significant reduction in IL-6 expression when compared to the group that received only 0.5% moxifloxacin. In contrast to the solvent-only group, the group treated with a combination of 0.5% moxifloxacin and 20 µg/mL EGCG exhibited a decrease in IL-6 expression. This finding indicates that the administration of EGCG alone is insufficient in reducing IL-6 expression in the Pseudomonas aeruginosa keratitis model, necessitating the presence of 0.5% moxifloxacin.

DISCUSSION

The process of wound healing on the cornea is both unique and complex. In contrast to other organs, the cornea is characterized by its avascular nature. Additionally, the cornea is distinguished by its transparency, which is important to its functionality. The preservation of transparency is a crucial aspect in wound healing and in preventing excessive fibrotic processes. In the case of Pseudomonas keratitis, the distinctive corneal lesion is a ring-shaped abscess within a corneal ulcer. The aforementioned lesions exhibit a rapid development and frequently culminate in corneal perforation, ultimately resulting in the loss of vision.

Table 1. Mean of clinical score and normality test

<table>
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<td>Mean (SD)</td>
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<th>Mean (SD)</th>
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Figure 3. The neutrophils count (1) and IL-6 expression (2) in areas of corneal keratitis: high density of neutrophil and IL-6 expression in K1 group (combined NaCl 0.93% and benzalkonium chloride 0.1% (A), less density of neutrophil and IL-6 expression in K2 group (moxifloxacin 0.5%) (B) and lesser density of neutrophil and IL-6 expression in K3 group (combined moxifloxacin 0.5% and EGCG 20 µg/mL) (C) (immunohistochemical staining, 400x magnification).
phospholipase, and hemolysin, which have the effect by changing the structure of the cornea and activating endogenous corneal proteases. These bacteria also have the ability to weaken the host response by interfering with important proteins and mediators, such as IgA and IgG, interleukins, complement, and gamma interferon (IFN-γ).\(^7\)

The process of corneal stromal wound healing is a multifaceted phenomenon that takes place in order to reinstate the optical clarity of the damaged cornea. This process entails the prompt initiation of programmed cell death in keratocytes, subsequently leading to the activation, proliferation, migration, and transformation into myofibroblasts. Myofibroblasts exhibit contractile properties in order to facilitate wound closure, while also releasing extracellular matrix components and proteinases to facilitate the degradation of the wound. The proteinases that are produced have the ability to break down the basement membrane, facilitating the passage of cytokines originating from the overlying epithelium. In the context of wound healing, immune cells are known to penetrate the site of injury with the purpose of eliminating cellular debris and mitigating the risk of infection. The basement membrane undergoes a gradual process of regeneration, leading to the disappearance of myofibroblasts and immune cells, reabsorption of the aberrant matrix, and subsequent restoration of corneal transparency.

The corneal wound healing process encompasses several distinct stages, namely homeostasis, inflammation, proliferation, and remodelling. The mechanism under consideration encompasses the participation of many growth factors, cytokines, and proteases that are synthesized by epithelial cells, keratocytes in the stroma, inflammatory cells, and lacrimal gland cells.\(^14\)\(^22\)

The homeostatic process initiates promptly after an injury, typically within minutes, with the activation of platelets. Subsequently, it progresses with the rapid synthesis of fibrin due to the sequential activation of the coagulation cascade. During this stage, neutrophils have the ability to increase the expression

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Figure 4. The neutrophils count among the negative control (solvent) group, moxifloxacin 0.5% and the combination of moxifloxacin 0.5% and EGCG 20 µg/mL. Significant if \(p<0.05\); \(*p=0.05\), \(**p=0.032\), \(***p=0.140\).

Figure 5. The IL-6 expression among the negative control (solvent) group, Moxifloxacin 0.5% and the combination moxifloxacin 0.5% and EGCG 20 µg/mL. Significant if \(p<0.05\); \(*p=0.016\), \(**p=0.191\), \(***p=0.064\).

In *Pseudomonas aeruginosa* keratitis, the process of developing corneal ulcers is faster, because *Pseudomonas aeruginosa* produces elastase and alkaline protease which can destroy corneal collagen. Collagen and corneal structures are damaged as a result of proteases produced by bacteria and the activation of corneal fibroblasts.\(^21\) *Pseudomonas aeruginosa* also produces exotoxins such as exotoxin-A,
of macrophages, T cells, TNF-α, IL-8, IL-1, and IL-6, which are involved in the process of phagocytosis of bacteria, pathogens, and cellular debris. During the initial stages of injury, there is a notable migration of tissue macrophages originating from monocytes inside the bloodstream. These macrophages exhibit ongoing phagocytic activity, while also attracting and stimulating the activation of fibroblasts, keratinocytes, and endothelial cells. The proliferative phase commences on the third day following injury, initiating the formation of granulation tissue through re-epithelialization, fibroblast proliferation, synthesis of type III collagen, and the development of vascularized tissue. Additionally, angiogenesis takes place during this phase. The aforementioned process is distinguished by the conversion of granulation tissue into a durable form of scar tissue.

All of the aforementioned procedures align with the findings of this investigation, wherein the examination of the anterior portion of rats’ eyes on the third day revealed the presence of keratitis in all samples that were infected. Moreover, these samples exhibited clinical scores of 2 and 3. A clinical value of 2 was assigned when the turbidity partially or fully obscured the pupil, whereas a score of 3 was given when opacities encompassed the entire front segment. If the process is not halted, it will result in the development of chronic inflammation and subsequent tissue degradation, characterized by a progressive deterioration of the cornea and escalating clinical scores.

The clinical score results align with the assertion that antibiotics have the capacity to eradicate germs, hence diminishing the bacterial-induced collagen breakdown. Nonetheless, the ongoing deterioration of corneal collagen persists as a consequence of corneal fibroblast activation, even in the absence of bacterial presence. The potent inflammatory response triggered by the toxin generated by P. aeruginosa hampers the efficacy of antibiotic treatment. The administration of antibiotic medication has the potential to rapidly eradicate germs, however, it is important to note that damage to the cornea may persist despite this treatment. This has been supported by previous studies.

**Effectiveness of EGCG to neutrophils count in keratitis P. aeruginosa**

In a research model of inflammatory angiogenesis, the inhibition of neutrophil-mediated angiogenesis was seen in vivo following the oral administration of EGCG and green tea extract. EGCG has been found to possess inhibitory properties against neutrophil elastase. The findings of the study indicate that micromolar EGCG effectively reduced the activity of reactive oxygen species and hindered the death of activated neutrophils. Furthermore, it significantly impeded the chemotaxis of neutrophils generated by chemokines in an in vitro setting. The findings of this study suggest that EGCG possesses substantial anti-inflammatory properties and may hold promise as a therapeutic agent (Nishida et al, 2021). EGCG demonstrates inhibitory effects on metallo-elastase and serine-elastase enzymes, which are released by macrophages (MMP-12) and neutrophils, respectively. EGCG has been found to exhibit a reduction in neutrophil transmigration across the endothelial cell monolayer, as well as a drop in the levels of several indicators associated with oxidative stress. The compound known as EGCG has the ability to regulate neutrophils by influencing their reactive oxygen species (ROS) activity, apoptosis, and migration in laboratory settings. These effects have implications for the resolution of inflammation, which is often accompanied by processes such as angiogenesis and fibrosis.

The findings of this study revealed a reduction in neutrophil count upon administration of the combination of moxifloxacin and EGCG. The addition of EGCG could reduce the absolute neutrophils count of blood lower than standard therapy. O’Callaghan et al, 2019 stated that EGCG as a polyphenol has a good antioxidant effect and plays a role in shortening the duration of inflammation by radical scavenging of nitric oxide (NO) so that NO levels quickly decrease which causes migration of neutrophils to the wound area to decrease and the inflammatory process becomes faster in pulp inflammation. with mechanical injury.

Hoffman et al, 2020 conducted a study on zebrafish and reported the presence of a confirmed mechanism of action of EGCG that leads to a reduction in neutrophil responses, including accumulation, travel speed, and signaling distance following injury. The findings of this study indicate a notable decrease in the local accumulation of neutrophils with high migratory speed following injury (n=33 cells, v=0.020 μm/s, d=37.8 μm). This decrease was observed after treatment with two different doses of EGCG: 300 μM (n=22 cells, v=0.013 μm/s, d=39.5 μm) and 600 μM (n=18 cells, v=0.008 μm/s, d=9.53 μm). EGCG has the ability to impede the chemotaxis of rat neutrophils towards cytokine-induced neutrophil chemoattractant-1 (CINC-1). This inhibitory effect was observed to be dependent on the concentration of EGCG. The chemotaxis of neutrophils produced by CINC-1 was found to be hindered by EGCG when administered at concentrations over 15 microg/mL. Additional findings indicate that EGCG has the ability to directly inhibit the infiltration of neutrophils, without exerting indirect effects such as suppressing the production of chemokines in inflammatory sites.

The impact of inhibiting EGCG on the migration of CD11b is observed in several immune cell types, including neutrophils, monocytes, NK cells, and CD8(+) T cell subsets. This inhibition plays a crucial role in diminishing the migration of neutrophils from the peripheral circulation to the site of inflammation. EGCG exhibits significant neutrophil activity both in laboratory settings (in vitro) and in living organisms (in vivo). This suggests that EGCG, as an orally administered pharmacological agent, has the potential to be an effective preventive treatment for individuals who are at risk of experiencing inflammation-related conditions, such as pulmonary fibrosis and tumor neoangiogenesis.

**Effect of EGCG on IL-6 expression in keratitis P. aeruginosa**

Chen et al, 2015 stated there exists a correlation between the presence of IL-6 and the recruitment of neutrophils in cases of bacterial keratitis. The cytokine known as interleukin-6 (IL-6) is of significant importance in the regulation of leukocyte recruitment inside the avascular cornea.
The immunological characteristics linked to the development of bacterial keratitis, specifically involving *Pseudomonas aeruginosa*, are characterized by higher levels of IL-8 compared to IL-6 in patients with bacterial keratitis. Additionally, patients with Gram-negative keratitis, including *Pseudomonas aeruginosa*, exhibit elevated levels of IL-8, IL-6, and IL-1β, along with an increased frequency of circulating CD3−CD56+ NK cells. According to another study, it has been proposed that IL-6 and IL-8 are cytokines that have a role in the immune-mediated ocular injury. This finding aligns with the results reported in a previous study, wherein it was shown that clinical scores for corneal damage were consistently rated as 2 and 3 across all treatment groups. The clinical ratings assigned were 2 when the opacities partially or fully obscured the pupil, and 3 when the opacities entirely obscured the entire anterior segment.

Zwolak et al., 2021 examined the occurrence of human bacterial keratitis on the human cornea in vivo. Their findings indicate a notable association between the infiltration of dendritic cells in the corneal stroma and elevated levels of interleukin-1β, interleukin-6, and interleukin-8 in tears. The findings indicated a decrease in cytokine concentrations and a reduction in the infiltration of dendritic cells following the intervention. There is a potential correlation between the simultaneous presence of IL-1β, IL-6, and IL-8 and the occurrence of immune system-mediated corneal damage in individuals infected with both Gram-negative and Gram-positive bacteria. This suggests that the levels of pro-inflammatory cytokines may exhibit temporal variations, with higher concentrations observed during the early stages of infection and a subsequent decrease during the later stages of infection or following treatment.

Human epithelial cells infected with Gram-negative bacteria exhibited evidence of IL-6 and IL-8 participation in the immune-mediated corneal injury. Additionally, the findings of this study indicate that the levels of IL-8 and IL-6 were elevated in the tears of individuals diagnosed with keratitis. Following a targeted intervention, researchers observed a reduction in the concentration of pro-inflammatory cytokines within tears and a decrease in the presence of polymorphonuclear cells (PMNs) on the ocular surface.

The results of this study showed a significant decrease in IL-6 after administration of EGCG compared to the control group which was only given solvent. Ahmed et al. (2008) stated that EGCG inhibited IL-1β-induced IL-6 production and transsignaling in RA synovial fibroblasts by inducing alternative splicing of gp130 mRNA, resulting in increased sgp130 production. EGCG also significantly prevented the release of H2O2, NOS (nitric oxide synthase) and 8-isoprostane, and reduced interleukin (IL)-1β, IL-2, and IL-6 levels. This study also concluded that there is a protective effect of EGCG against stress-induced liver injury (RS) and immunosuppression. EGCG significantly reduces stress hormone release to a weak stress response.

EGCG apart from having anti-inflammatory and antioxidant effects also has antibacterial effects. EGCG can cross-link with many proteins, and exerts various anti-bacterial activities by destroying microbial cytoplasmic lipids and proteins. EGCG at certain doses is also stated to provide protection against deadly polymicrobial infections and significantly reduces the number of bacteria, especially in the liver and lungs. Mechanism of action of EGCG is to damage the activity of the cytoplasmic membrane pump, which results in increasing intracellular antibiotic concentrations, so the results of this study support the possibility of using EGCG as an adjuvant in antibacterial therapy. EGCG can cross-link with various proteins and result in the induction of changes in protein confirmation. This shows that EGCG is a strong protein remodeling agent.

EGCG binds competitively to the STAT3 SH2 domain, inhibiting STAT3 phosphorylation and signaling. STAT3 plays an important role in immune regulation including IL-6, IL-10, IL-21, and IL-23 receptors, which in turn play a role in the autoimmunity of the hyper-inflammatory condition COVID-19. A high level of IL-6 in COVID-19 appears to be a major prognostic factor for a worse outcome. In conclusion, EGCG is very promising in the treatment of COVID-19 because it is a strong inhibitor of the STAT3 pathway. EGCG promotes significant long-term protection against sepsis experimentally because administration of 4 mg/kg significantly saved mice from sepsis, thus supporting the therapeutic potential of EGCG in the clinical management of human septicemia. EGCG attenuated serum levels of IL-6 and HMGB1 in rats and 10 μM was expressed reduced IL-6, TNF-α and nitric oxide secretion in primary murine peritoneal macrophages stimulated with HMGB1.

The results showed that there were significant differences in the expression of IL-6 and the number of neutrophils between the group treated with solvent only and the group treated with a combination of moxifloxacin 0.5% and EGCG 50 μg/mL. However, there was no significant difference among the solvent-treated group and the group that was only treated with 0.5% moxifloxacin, and the group that was only treated with 0.5% moxifloxacin and the group that was treated with a combination of 0.5% moxifloxacin and EGCG 50 μg/mL. The results were also more extensive damage to the corneal tissue in the solvent-only *Pseudomonas aeruginosa* keratitis group which was characterized by the highest mean percentage of IL-6 expression and neutrophils count among the three treatment groups. It shows that the combination therapy of moxifloxacin 0.5% and EGCG 50 μg/mL has the potential to inhibit IL-6 expression and the number of neutrophils produced by damage to the corneal epithelium, due to mechanical trauma to the epithelium and invasion of *Pseudomonas aeruginosa* bacteria. Thus, it is hoped that in addition to standard antibiotic therapy (moxifloxacin), EGCG can be used as a therapy to reduce the inflammatory response thereby preventing the formation of corneal opacities.

**CONCLUSION**

EGCG reduced neutrophils count and IL-6 expression levels in the combined EGCG and moxifloxacin group compared to the moxifloxacin group and solvent group in animal models of *Pseudomonas aeruginosa* keratitis. The findings of this
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study proved that the EGCGr have potency as an adjuvant to suppress inflammation in Pseudomonas aeruginosa keratitis.

CONFLICT OF INTEREST

The authors affirmed that there were no conflicts of interest in this study.

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ETHICAL CLEARANCE

This study has obtained ethical clearance from Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya with reference letter number 2.KEH.163.12.2022.

AUTHOR CONTRIBUTION

All authors contributed equally in this research and publication of this manuscript.

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