Expression of Matrix Metalloproteinase-8 (MMP-8) and Tissue Inhibitors of Metalloproteinase-1 (TIMP-1) after cryotherapy in Aspergillus flavus keratitis at Dr. Soetomo General Academic Hospital, Surabaya, Indonesia

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ABSTRACT

Background: Fungal keratitis is one of the most difficult forms of microbial keratitis to treat successfully. Aspergillus flavus is a type of filamentous fungus that often causes suppurative keratitis. Complications that often arise from fungal keratitis are corneal perforation and blindness due to scar tissue formation.

Methods: Aspergillus flavus was injected into the corneas of 28 Sprague Dawley rats intrastromal and divided into 4 groups, with each group consisting of 7 rats, fungal injection into the cornea as a fungal injection without therapy, natamycin therapy, cryotherapy, and combination therapy. Therapy was given five days after the fungal injection into the cornea and the formation of keratitis and corneal central thinning. After four days of therapy, the eyes were enucleated to determine the effect of cryotherapy on MMP-8 and TIMP-1 expression in the cornea with an immunohistochemistry (IHC) staining examination. The differences in MMP-8 and TIMP-1 expressions between groups were analyzed using the Kruskal Wallis test and Mann-Whitney post hoc test with a significant p < 0.05. Data were analyzed using SPSS version 26 for Windows.

Results: The highest MMP-8 expression was found in the natamycin therapy group (3), and the lowest was in the negative control group (2). There were no significant differences in MMP-8 expression in the 4 groups (p=0.482). The highest TIMP-1 expression was found in the negative control group (8) and the lowest was in the cryotherapy group (2). There were significant differences in TIMP-1 expression in the 4 groups (p=0.002).

Conclusion: It was found that the expression of TIMP-1 and MMP-8, although the last one was not significant, were lower in the cryotherapy group than in the non-cryotherapy group.

Keywords: Fungal Keratitis, Aspergillus flavus, Cryotherapy, MMP-8, TIMP-1, Infectious Disease.


INTRODUCTION

Fungal keratitis, also known as keratomycosis, is a corneal infection caused by one of several pathological fungi that attack the cornea. This disease usually progresses slowly.¹ In the early stages, patients with fungal keratitis have milder signs and symptoms of inflammation than those with bacterial keratitis, with less conjunctival injection and more prominent pain. Patients with fungal keratitis frequently present with severe symptoms. A retrospective study discovered that fungal keratitis was 5.86 times more likely than bacterial keratitis to cause corneal perforation and irreversible corneal lesions.²³

Natamycin is the most commonly prescribed treatment for filamentous fungi and the only antifungal approved by the US Food and Drug Administration (FDA). However, studies have shown that A. flavus has a higher MIC (minimum inhibitory concentration) than other Aspergillus species.⁴ For example, the MIC for natamycin in A. fumigatus is 4 g/ml, whereas the MIC in A. flavus is 64 g/ml. This suggests that natamycin has limited access to ergosterol in the cell membrane of A. flavus.⁴

Cryotherapy in cases of keratitis has not been widely reported. The study of cryotherapy as a treatment for fungal keratitis in experimental rabbit animals showed a faster healing time compared to the control group. Moreover, scanning electron microscopy (SEM) examination revealed fungal cell damage, cell shrinkage, and hyphae damage.⁵

Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases that regulate cells via various mechanisms, one of which is extracellular matrix proteolysis. In human and animal studies of keratitis models, the expression of MMP-1, -2, -8, -9, and -13 was higher. MMP-8 is involved in neutrophil migration in the corneal stroma.⁶

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Tissue Inhibitors of Metalloproteinase (TIMP) is a multifactorial molecule that inhibits MMP activity. TIMP-1 inhibits MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, and -16. TIMP-1 expression is upregulated in a profibrotic environment, implying that TIMP is involved in extracellular matrix proteolysis inhibition. Stoichiometrically, TIMP binds to MMP in a 1:1 ratio, so a change in TIMP concentration will directly affect MMP activity. TIMP-1 expression is upregulated with fungal keratitis caused by A. flavus. Based on the mentioned above, the authors hypothesized that the cryotherapy group would have lower MMP-8 and TIMP-1 expression than those who were not treated with cryotherapy.

**METHODS**

This is a randomized posttest-only control group study with an experimental design. The experimental rats of the *Rattus norvegicus* species will be divided into four groups: the negative control group, the therapeutic natamycin group, cryotherapy, and combination therapy groups. The expression of MMP-8 and TIMP-1 was then compared between the four groups.

The inclusion criteria for the experimental unit of this study were *Rattus norvegicus* rats aged 6-8 weeks with a weight of 250-300 grams. The mice were in good health and looked active, and the eyes were healthy on external examination. Exclusion criteria for the experimental unit of this study include animals declared by a veterinarian to have a disease or the potential to transmit the disease during evaluation. The criteria for dropping out of the test were sick, dead rats, and complications such as corneal perforation and prolapse of the contents of the eyeball.

28 rats met the inclusion criteria. A handheld slit lamp was used to examine the anterior segment. The rats were divided into four groups, each with seven rats. The right eye was given topical anesthesia with 2% tetracaine hydrochloride eye drops. Injections of ketamine hydrochloride 50-75 mg/kg and xylazine 5-8 mg/kg is given. Each group of rats received a solution of *A. flavus* spores with a concentration of 5x10⁶ CFU/ml intrastromal (tangentially) as much as 0.2 ml and a subconjunctival triamcinolone acetonide injection of 0.2 ml. After that, apply Levofloxacin eye drops. After the infiltrate appeared five days later, the treatment group received natamycin 5% eye drops therapy four times daily, cryotherapy, and combination therapy. In the cryotherapy group, CO2 was used with a frozen tissue diameter 1 mm larger than the cryotherapy probe cross-section, temperatures ranging from -50°C to -60°C, and freezing times ranging from 7 to 8 seconds in the right eye. On day 9, enucleation was performed.

The corneal lesion from enucleated tissue was submitted for the paraffin section. Immunohistochemistry (IHC) was applied to evaluate MMP-8 and TIMP-1 expression. Prior to IHC, the paraffin-embedded tissue was sectioned in 4 μm thickness. The sections were deparaffinized with xylene, dehydrated with ethanol, and deoxidized with methanol. The sections were prepared in a decloaking chamber deoxidized with methanol. The sections were then mounted with a permount visualization for 5 minutes. The sections were processed for diaminobenzidine (DAB) staining and then incubated in a temperature-resistant medium and observed under a light microscope (Olympus CX41).

The collected data will be analyzed using SPSS version 26 for Windows. The Kruskal-Wallis test is used to conduct the analysis. The post hoc Mann-Whitney test was used to compare the expression strength was analyzed and graded based on the percentage of positive cells and intensity of immunoreactivity. The positive cells were stained light brownish to chocolate–brown, and the intensity of the immunoreactive products was scored under a light microscope as follows: no expression, 0; minimal reaction, 1; moderate reaction, 2; and strong reaction, 3. The positive ratio was scored as follows: no positive cells, 0; positive cells <10%, 1; positive cells 11–50%, 2; positive cells 51–80%, 3; positive cells >80%, 4. The two scores were multiplied, and the immunoreactive score (IRS) (values from 0–12) was determined as follows: 0-1 (−), 2–3 (+), 4–8 (++), and >9 (+++).

The expression of MMP-8 and TIMP-1 were located in the cytoplasm. The expression strength was analyzed and graded based on the percentage of positive cells and intensity of immunoreactivity. The positive cells were stained light brownish to chocolate–brown, and the intensity of the immunoreactive products was scored under a light microscope as follows: no expression, 0; minimal reaction, 1; moderate reaction, 2; and strong reaction, 3. The positive ratio was scored as follows: no positive cells, 0; positive cells <10%, 1; positive cells 11–50%, 2; positive cells 51–80%, 3; positive cells >80%, 4. The two scores were multiplied, and the immunoreactive score (IRS) (values from 0–12) was determined as follows: 0-1 (−), 2–3 (+), 4–8 (++), and >9 (+++).

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**Figure 1.** Clinical development from day1-5. A) On day 1, the cornea had a thin central infiltrate; B) On day 2, thickened infiltrates, conjunctival hyperemia, and conjunctival chemosis were observed; C) On day 3, the infiltrate thickened, conjunctival hyperemia and conjunctival chemosis developed; D) On the third and fourth day, there was no significant difference in the clinical picture; and E) On Day 5, there was central corneal thinning.
test is used to compare groups due to the analysis results are significant. The level of significance was set at $p < 0.05$.

**RESULTS**

This study used an experimental unit of the right eye cornea of 28 *Rattus norvegicus* rats obtained from a rat farm under the supervision of a veterinarian, weighing 150 grams and aged 6 weeks.

The research was conducted at the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya.

On the first day, a hand-held slit lamp revealed a thin infiltrate as deep as the epithelium. On the second day, the central corneal infiltrate became thicker, and the conjunctiva was slightly hyperemic. On the third day, a thick infiltrate as deep as the stroma developed, resulting in conjunctival hyperemia and chemosis.

On the fourth day, there was central thinning of the cornea from keratitis, deep stromal infiltrate, chemosis and conjunctival hyperemia. The infiltrate was gray-white and well-defined, with no satellite lesions or hypopyon in the anterior chamber. On day 4, after natamycin therapy, cryotherapy, and combination therapy on enucleation, no clinical changes were visible using a hand-held slit lamp (Figures 1 and 2).

The IHC MMP-8 examination revealed that the median value of the negative control group was 1, with an interquartile deviation (IQD) of 2.5. The median value of the natamycin-treated group was 3, and the IQD was 1.5. The cryotherapy group had a median value of 1 and an IQD of 0.5. The combination group had a median value of 2 and an IQD of 3.5. The Kruskal-Wallis test revealed no significant differences in MMP-8 expression intensity between the four groups ($p=0.482$) (Table 1).

The IHC TIMP-1 examination revealed that the median value of the negative control group was 8, with an IQD of 1. The natamycin therapy group had a median value of 6 and an IQD of 2.5. Cryotherapy had a median value of 2 and an IQD of 1. The combined therapy group had a median value of 3 and an IQD of 3.5. The Kruskal-Wallis test revealed a significant difference in TIMP-1 expression intensity between the four groups ($p=0.002$) (Table 2). A Post-Hoc Mann-Whitney test revealed a significant difference in TIMP-1 expression intensity ($p<0.05$) in all groups except the negative control and natamycin-treated groups ($p=0.202$) and the cryotherapy group and the combination therapy group ($p=0.639$) (Table 2).

**DISCUSSION**

Fungal keratitis, also known as keratomycosis, is an infectious disease of the cornea caused by various pathological fungi that can invade the ocular surface. The most common fungi that can cause keratomycosis are *Candida spp*. In addition, it can also be caused by *Aspergillus spp.*, *Fusarium spp.*, *Cladosporium spp.*, *Curvularia*, and *Rhizopus*. Fungal keratitis can infect people with uncontrolled
systemic disease. Clinical manifestation of this disease is characterized by severe inflammation in the form of a grayish-white or yellowish-white infiltrate at the base of the ulcer, a region of elevated ulcer with irregular edges, feathery margins, a rough and dry surface, satellite lesions, and hypopyon. In general, treatment options include medical and surgical procedures. Medical treatment can be delivered topically or systemically. Natamycin 5% is the only commercially available topical antifungal. Medical and surgical management can be combined if the clinical condition does not improve. In addition to other surgical procedures, corneal scrapings can be performed to increase the penetration of topical therapy. This technique, however, is no longer recommended because it can deteriorate vision after three months. In addition, conjunctival flaps and keratoplasty, both lamellar and penetrating, can be performed.

On the fifth day of observation, clinical changes were observed after injection of the fungus Aspergillus flavus using a hand-held slit-lamp, specifically thinning of the cornea in the center of the ulcer. There were no satellite lesions or hypopyon in the anterior chamber, and the infiltrate was grayish white with well-defined borders. The hand-held slit-lamp revealed no clinical changes until day 4 after cryotherapy when enucleation was performed. According to the previous study, the effect of cryotherapy on the healing of fungal keratitis was observed using a slit-lamp starting on day 15 after cryotherapy.

According to several studies, cryotherapy can reduce the infiltration of inflammatory cells in the tissue. A study by Hurme T et al. stated that the number of neutrophils and macrophages in the muscle tissue of animals that had undergone trauma treatment followed by cryotherapy was lower than in the control group. Another study found that cryotherapy can reduce tissue inflammation. This is indicated by a decrease in the number of macrophages and granulocytes compared to controls.

According to a qualitative review by Bleakley CM and Davidson GW found that cryotherapy could reduce neutrophil infiltration that occurs consistently after 24 hours. In fungal keratitis, the expression of IL-1β, -6, -8, and -17 was found to be elevated. Activation of IL-1β, IL-17, and neutrophils can increase the expression of MMPs, especially MMP-8 and MMP-9. In keratitis, MMP is the cause of poor wound healing. In the study by Yuan X et al., an increase in the expression of MMP-8, -9, and -13 in fungal keratitis was noted. During inflammation, keratocytes in the cornea express growth factors such as TGF-β1. TGF-β1 plays a role in wound healing through the proliferation and differentiation of fibroblasts, which results in the formation of myofibroblasts and the deposition of extracellular matrix, which induces fibrosis during the wound closure process.

MMP-8 (neutrophil collagenase) is a collagenease secreted by PMNs, macrophages, fibroblasts, epithelium, endothelial cells, and keratocytes. Collagen is the most abundant structural protein in the human body and a key component of the extracellular matrix. Type I collagen predominates in the corneal stroma, with other types of collagen present in smaller amounts. In this study, there were no differences in MMP-8 expression between the negative control group, natamycin therapy, cryotherapy, and combination therapy observed with IHC staining. In a previous study, it was stated that there was no significant difference in the expression of MMP-9 in fungal keratitis due to *A. flavus* in the cryotherapy and non-cryotherapy groups.

In this study, there were significant differences in the intensity of TIMP-1 expression except in the negative control group and the natamycin group and between the cryotherapy group and the combination therapy group. TIMP expression mechanisms were discovered to be MMP-dependent and MMP-independent. In the MMP-dependent mechanism, the function of TIMP-1 in wound healing is to induce the production of fibroblasts by inhibiting MMP expression so that it can reduce the degradation of the extracellular matrix by MMP. However, in another study, TIMP-1 was found to have MMP-independent functions, such as mediating the interaction of CD63 and β1 integrins with fibroblasts, inducing Smad2/3 and β-catenin translocations, and inducing mRNA expression from collagen type I and III.

In the negative control group, the median level of TIMP-1 expression was eight. TIMP-1 is an MMP inhibitor that inhibits MMP-14, -15, -16, 19, and -24, except for membrane-type MMP (MT-MMP). TIMP-1 expression can be an MMP-dependent or MMP-independent direct effect. In fungal keratitis, in addition to MMP-8, there was an increase in the expression of MMP-9 and MMP-13. The negative control group in this study had a median MMP-8 expression of 1. However, this study did not examine the expression of other types of MMP. In the MMP-dependent mechanism, if there was no increase in MMP-8 expression in this study, but TIMP-1 as an endogenous inhibitor increased, it can be assumed that there was an increase in other types of MMP so that TIMP-1 can bind to MMP with a 1:1 complex.

The median expression of TIMP-1 in the cryotherapy group was 2. Fungal keratitis may amplify the expression of MMP-8, -9, and -13. In previous studies, it was stated that cryotherapy could reduce the expression of IL-1β and TNF-α, reducing the expression of MMP-8, -9, and -13. The decrease in MMP causes a decrease in TIMP-1 expression of the MMP-dependent mechanism. This can cause TIMP-1 expression in the cryotherapy group to be lower than in the negative control group.

Natamycin can stimulate IL-1β secretion in dendritic cells and macrophages. There are two activation pathways of pro-IL-1β to become IL-1β. First, via an NF-κB-dependent mechanism. This process requires activation of TLR, C-type lectin, and TNFR. The second pathway is to activate caspase-1. Natamycin requires an NLRP3 inflammasome and an ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) to activate caspase-1. Natamycin can destabilize cell membranes and cause cells to release potassium, which activates the NLRP3 inflammasome. Furthermore, natamycin can induce phagocytosis, which can release lysosomal proteases, which can activate cathepsin and modulate NLRP3 activation.

(continued...)
In the natamycin group, the median MMP-8 expression was 3. Although not significantly different, MMP-8 expression in both groups was higher than in the negative control group (median=1) and cryotherapy group (median=1). The natamycin therapy group had a median TIMP-1 expression of 6. TGF-β1 expression was increased by NLRP3 expression without NF-kB activation. NLRP3 stimulates increased expression of TGF-β1 through increased secretion of IL-1β. Increased neutrophil infiltration caused by IL-1β contributes to tissue damage by releasing proteolytic enzymes, reactive oxygen species (ROS), and MMP-9 enzymes. Furthermore, neutrophils secrete MMP-8, which assists neutrophil migration to wound edges. Increased MMP-8 expression in the cornea causes extracellular matrix degradation, which leads to stromal collagen denaturation or destruction, disrupting tissue remodeling and contributing to fibrosis. The profibrotic environment induces fibroblasts to express TIMP-1. This may influence the increase in MMP-8 and TIMP-1 expression during natamycin therapy. While in the combination therapy group, the median expression of MMP-8 and TIMP-1 was between the natamycin and cryotherapy groups, it can be assumed because of the competition in the therapeutic mechanism of natamycin and the anti-inflammatory effect of cryotherapy. The limitation of this study is that IHC examination was only performed once 9 days after fungal infection or 4 days after therapy administration. Therefore, the dynamic of MMP-8 and TIMP-1 expression could not be observed. Other examination methods, such as the Enzyme-linked Immunosorbent Assay (Elisa), are needed to determine the peak expression time and the decrease in the expression of MMP-8 and TIMP-1 in experimental animals with different time durations.

CONCLUSION

In this study, the expression of MMP-8 and TIMP-1 was lower, though not significantly, in the fungal keratitis group that received cryotherapy compared to the group that did not receive cryotherapy.

CONFLICT OF INTEREST

There is no conflict of interest regarding the manuscript.

ETHICS CONSIDERATION

Ethics approval has been approved by the Ethics Committee, Faculty of Medicine Universitas Airlangga, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, prior to the study being conducted.

FUNDING

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AUTHOR CONTRIBUTION

All authors equally contribute to the study from the conceptual framework, data acquisition, and data analysis until reporting the study results through publication.

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