Cryotherapy Has No Significant Effect on MMP-9 and TGF-β1 Expression in Fungal Corneal Ulcer

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RESEARCH ARTICLE

Abstract

BACKGROUND: Usually, surgical intervention is needed to eradicate the fungal microorganism that cause fungal corneal ulcers. However, since surgical intervention is invasive, the latest technology uses cryotherapy in treating it. Cryotherapy plays a vital role in the wound healing process. We aimed to evaluate is to study the decreased expression of matrix metalloproteinase 9 (MMP-9) and transforming growth factor β1 (TGF-β1) in fungal corneal ulcers after the administration of cryotherapy.

METHODS: Aspergillus flavus fungus was injected to the intrastromal corneas of all Sprague Dawley rats. The rats were divided into four groups, the first group was not given any therapy, the second group was given topical natamycin therapy, the third group was given cryotherapy, and the fourth group was given a combination between cryotherapy and topical natamycin therapy. Therapy was given after five days of follow up on the formation of a corneal ulcer. After four days of therapy, the eyes were enucleated to determine MMP-9 and TGF-β1 expression.

RESULTS: The result in the third group showed lower MMP-9 expression (20.0±10.0% cells per field of view) compared to the second group (40.0±20.0% cells per field of view) and the fourth group (30.0±25.0% cells per field of view), but had the same MMP-9 expression value as the first group. There was no significant difference in MMP-9 expression between the four groups (p=0.356). The third group reduced more TGF-β1 expression (10.0±12.50% cells per field of view) compared to the fourth group (30±27.5% cells per field of view) and the first group (30±32.5% cells per field of view). There was also no significant difference in TGF-β1 between the four groups (p=0.315).

CONCLUSION: There is no significant difference in the expression of TGF-β1 and MMP-9 after the cryotherapy treatment.

KEYWORDS: corneal ulcer, cryotherapy, MMP-9, TGF-β1

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Introduction

Fungi are one of the most challenging keratitis-causing organisms to diagnose and treat. Fungal keratitis is a cause of eye morbidity and blindness. Fungal keratitis is common in tropical climates, with an incidence of between 6% to 20% concerning all microbial keratitis cases. Generally, fungal keratitis causes corneal ulcers in developing countries, including South India (44%), Bangladesh (36%), Ghana (37.6%), and Nepal (17%). Each year, an estimated 80,000 total cases are due to fungal corneal infections in India. (1,2) The most common fungal organisms were Aspergillus (27%–64%), Fusarium sp. (6%–32%), and Penicillium sp.
Surgical intervention is needed to treat fungal corneal ulcers in some cases. However, since surgical intervention is quite invasive, another method is necessary. The recent technology developed cryotherapy to treat fungal corneal ulcers. Cryotherapy is tissue destruction using a cold temperature valuable substance for reducing inflammation and the number of hyphae. Cryotherapy causes denaturation and degradation of cell proteins. The effect of cryotherapy can reduce the dissolution of the corneal stroma and significantly shorten the duration of healing. Cryotherapy inhibited the expression of transforming growth factor β1 (TGF-β1) secreted by macrophages and decreased TGF-β1 expression on the 3rd and 7th days after therapy. The decrease in macrophage infiltration affected the reduction of matrix metalloproteinase 9 (MMP-9) expression three days after the lesion’s cryotherapy.

Research on corneal ulcers using cryotherapy is important and necessary for biomolecular research development and plays a vital role in the wound healing process, however the amount is still limited. Therefore, this study aims to determine the effect of cryotherapy as an alternative therapy in corneal ulcers, comparing the expression of MMP-9 and TGF-β1 as part of the corneal wound healing process.

### Methods

#### Study Design and Subject

This study was an experimental research with a randomized post-test only control group design. The study was conducted at the Faculty of Veterinary, Universitas Airlangga, and Anatomical Pathology Laboratory of Dr. Soetomo General Hospital from February 2020 to June 2020. The Health Ethics Committee of Faculty of Veterinary, Universitas Airlangga approved this study (No. 2.KE.032.04.2020).

#### Subjects Treatment

The inclusion criteria of the study subjects were *Rattus norvegicus* ages 6 to 8 weeks old, with healthy eyes and a healthy body. The exclusion criteria are *Rattus norvegicus* with infectious disease. Twenty-eight rats met the inclusion criteria and divided into four groups, seven rats each. All groups consist of rats with *Aspergillus flavus* injection in the cornea. The first group was given only fungal injections without therapy, and act as the control group. The second group was given topical natamycin therapy. The third group was given cryotherapy. The fourth group was given combination therapy of topical natamycin and cryotherapy.

#### Sample Preparation

Anterior segment examination was performed using a handheld slit lamp. Topical anesthesia was administered with 2% tetracaine hydrochloride (pantocaine) eye drops in all right eyes, giving an injection of ketamine hydrochloride 50 mg kg⁻¹ to 75 mg kg⁻¹ and xylazine 5 mg kg⁻¹ to 8 mg kg⁻¹. Corneas were scratched three times on the cornea surface using a scalpel approximately 4 mm. All group rats in the right eye were injected with 0.2 mL Aspergillus flavus solution with a concentration of 5×10⁵ spores per eye intrastromal and 0.2 mL injection of triamcinolone acetonide subconjunctival to help fungal growth and to accelerate the occurrence of fungal infection with the field of operation narrowed: a sterile drape, attach eyelid speculum, irrigate the eyeball with sterile water, and put Levofloxacin eye drop to clean the conjunctival sac. Then the mice were treated for five days and observed every day using a handheld slit lamp and one drop of antibiotic Levofloxacin eye drop into the right eye as prophylaxis against bacterial infections.

After the infiltrates appeared within five days, the first group of rats was not given therapy, the second group of rats was given natamycin drops, in the third group of rats, cryotherapy was carried out with a frozen tissue diameter 1 mm larger than the cross-section of the cryoprobe, the temperature reached -50°C to -60°C with freezing time and between 7 s to 8 s in the right eye, while the fourth group of rats was given combination therapy of natamycin drops and cryo. This study used cryo super deluxe AA3 SL.NO 13113038 with a corneal probe placed in the cornea center at the defect area. Natamycin was given one drop as an antifungal eye drop in the right eye of the rats. On the 4th day after therapy, the eyeball was removed (enucleation), then the cornea was subjected to immunohistochemistry (IHC) staining for evaluating the percentage of positive cells. Periodic acid–Schiff (PAS) staining was performed and observed in all four groups in this study using 400× magnification for hyphae detection.

#### IHC Staining

After enucleation, the eyeball was put into a bottle with a 10% buffered formaldehyde buffer solution to inhibit tissue decay. After that, the eye tissue was taken to the anatomical pathology room for preparation making paraffin block. The immunohistochemistry (IHC) staining procedure was deparaffinized, rehydrated tissue section, reduced
nonspecific background due to peroxidase block, and was incubated slide in hydrogen peroxide for 10 min to 15 min. In heat induced epitope retrieval (HIER) buffers, the tissue was incubated in the digestive enzyme and heat designated slide for 45 min at 95°C.

Furthermore, the excel block was applied and incubated for 5 min to 10 min at room temperature to block nonspecific background staining. Antibody MMP-9 and TGF-β1 were applied and incubated. The excel link was applied and incubated for 15 min to 20 min at room temperature. The excel HRP was applied and incubated for 20 min at room temperature. The 3,3'-Diaminobenzidine (DAB) chromogen was applied for 5 min to 15 min, rinse the tissue, counterstain, dehydrate, clear in xylene, and coverslip.

Corneal expression of MMP-9 was evaluated by an immunohistochemical method with the polyclonal antibody (Cat No. bs-4593R, Bioss Antibodies, Woburn, MA, USA) diluted 1:200. Meanwhile, TGF-β1 expression in the cornea was evaluated using an immunohistochemical method with the monoclonal antibody TGF β1 (Cat No. ABIN2476751, GmbH Schloss, Rahe, Germany) diluted 1:100. MMP-9 and TGF-β1 were positively expressed on corneal stromal keratocytes. An anatomical pathologist and researcher carried out the assessment. The number of stromal keratocytes that react to MMP-9 and TGF-β1 antibodies was shown in brown color and was assessed quantitatively by a light microscope at 400× magnification of the stromal keratocytes.

**Statistical Analysis**
The collected data were analyzed using SPSS (IBM Corporation, Armonk, NY, USA) 12.0 version. If the data were normally distributed, the bivariate analysis used ANOVA. If the data distribution was not expected, the analysis used Kruskal-Wallis and Mann Whitney post hoc. Data were expressed in mean±SD with a significant level of $p<0.05$.

### Results

**Corneal Ulcer Forming on Rat Cornea**
On the first day, a thin epithelial-deep infiltrate was observed using a handheld slit lamp (Figure 1). On the second day, the infiltrate was getting thicker in the center of the cornea, the conjunctiva was slightly hyperemic. On the third day, there was a thick stromal-deep infiltrate, and conjunctival hyperemia and chemosis began. On the fourth day, there was no significant difference with the third day of the clinical picture of ulcers. On the fifth day, corneal thinning was at the center of the ulcer, stromal-deep infiltrates, chemosis,
and conjunctival hyperemia. As a result, infiltrates were grayish-white, well-defined, and there were no satellite lesions or hypopyon in the anterior chamber.

**Cryotherapy for Corneal Ulcer**

Cryotherapy was administered on day five, after injection of the fungus into the corneas of the rats' right eye. Cryotherapy was carried out 7 s to 8 s, or around 1 mm wide icicle formed the cryoprobe’s tip in freezing phase on corneal ulcers surface. From a handheld slit lamp saw no clinical changes until the 4th day after administering cryotherapy at enucleation time (Figure 2).

**Fungal Hyphae Identification Using Periodic Acid Schiff (PAS) Staining**

There was a positive PAS with an intact hypha and pink color in the stromal layer of the cornea in the control group. Several hyphae invaded the lens with infiltration of neutrophils and macrophages around it. Also, there was stromal edema and necrosis (Figure 3). There were fragmented hyphae in the natamycin therapy group in the stromal layer of the cornea, and several hyphae had invaded the lens. There was an infiltration of neutrophil cells, and macrophages reduced compared to the control group. In the cryotherapy and combination therapy groups, hyphae images were obtained with fragments breaking into small pieces and invading the lens, similar to the natamycin therapy group.

**Effect of Fungal Injection Treatment and Therapy on Corneal Tissue MMP-9 Expression**

The statistical test on the number of cells expressing MMP-9 showed that the median value of the control group was 20.00±42.50. The median value of the fungal injection and natamycin groups was 40.00±20.00. The median value of the fungal injection and cryotherapy group was 20.00±10.00, and the median value of the fungal injection and combination therapy group between natamycin and cryotherapy was 30.00±25.00. The Kruskal Wallis statistical test did not show any significant difference in the number of cells expressing MMP-9 between the four groups with

**Figure 2. Clinical features of the cornea after cryotherapy.** Shortly after cryotherapy, the cornea is still persistent (obtained corneal thinning [black arrow], and conjunctival chemosis [red arrow]) in C3 labeled mice (from fungal injection with cryotherapy group).

**Figure 3. PAS staining in a mouse animal model in the corneal layer.** A: Positive PAS staining in the control group, which shows the presence of intact fungal hyphae, which is pink color in the corneal stroma (black arrow) with inflammatory cell infiltration, edema, and stromal necrosis (yellow circle). B,C,D: No hyphae were detected in the cryotherapy, natamycin, and combination therapy groups, but there was an infiltration of inflammatory cells (black arrow) in the corneal stroma. White bar: 20 µm.
**Effect of Cryotherapy on MMP-9 and TGF-β1 in Fungal Corneal Ulcer (Permatasari D, et al.)**

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**Discussion**

In this study, no clinical change after cryotherapy, by handheld slit lamp, was until the enucleation time on the 4th day. According to previous study, cryotherapy effect on the fungal corneal ulcers healing was observed with handheld slit lamp after the 15th day. (5) Fungal hyphae and inflammatory cell infiltration were observed with PAS staining on the fourth group. Additionally, early inflammation is localized and involved two-thirds of the anterior stroma. Continuous inflammation of the deep stroma can cause total damage to the stroma resulting in necrosis. (7) Histopathology examination of fungal corneal ulcers can be found fungal filaments, inflammatory cells, and granulomatous inflammation. (8) Histopathological change from PAS staining on 15th day after cryotherapy resulted in epithelial cells growth, more regular stromal collagen fibers, and less fungal hyphae. (5)

After *Aspergillus fumigatus* inoculation intrastromally, which was observed on the 6th to 10th day, it was found that the fungi invaded the deeper stroma and destroyed the endothelial cells. On the 6th day, the treatment group show hyphae fragments break into small pieces. (9) Strain

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In the fungal injection and cryotherapy group, the mean value of MMP-9 was 5.36, while the mean value of combination therapy between natamycin and cryotherapy was 9.64. The Mann-Whitney statistical analysis found a significant difference in the number of cells expressing MMP-9 between the two groups with $p=0.049$ ($p<0.05$). Figure 4 showed that immunoreactive expressing MMP-9 in keratocyte stromal cells stained brown color shown with the black arrow.

### Effect of Fungal Injection Treatment and Therapy on Corneal Tissue TGF-β1 Expression

The statistical tests on the number of cells expressing TGF-β1 in the median value of the control group was 30.00±32.50. There were the median value results of the fungal injection and natamycin groups (10.00±14.00), the fungal injection and cryotherapy groups (10.00±12.50), the fungal injection and combination therapy group between natamycin and cryotherapy (30.00±27.50). Kruskal Wallis statistical test showed no significant difference in the number of cells expressing TGF-β1 between the four groups with $p=0.315$ ($p>0.05$). Figure 5 showed that immunoreactive expressing TGF-β1 in keratocyte stromal cells stained brown color shown with the black arrow.

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**Figure 4. IHC staining with polyclonal antibody MMP-9 on corneal stromal keratocytes.** A: Expression of MMP-9 in the corneal stromal keratocytes (black arrow) of the control group with the percentage of cells expressing MMP-9 30%. B: Expression of MMP-9 in the corneal stromal keratocytes in the cryotherapy group (black arrow). C: Expression of MMP-9 in the corneal stromal keratocytes of the combination therapy group between cryotherapy and natamycin (black arrow) with the percentage of cells expressing MMP-9 70%. White bar: 20 µm.
Aspergillus flavus causes opacity on stroma lamellae with PMN infiltration, can penetrate the intact Descemet membrane, and invade the anterior chambers of the eye. There were no significant differences in the four groups. In the control group, the median results were similar to the cryotherapy group. The limitation of this study was immunohistochemical examinations were carried out at one time on 9th day after fungi inoculation. It may cause a decrease in the number of cells expressing MMP-9 in the control group, which affects statistical results not significantly.

According to previous studies that examined the presence of MMP expression during the fungal keratitis process, the results showed that MMP-9 significantly increased in the observation period on days 1, 2, 3, 6 while it began to decrease on the 10th day and decreased significantly on day 14. Correspondingly, MMP-9 expression in a rabbit animal model was subjected to corneal wounds and observed 4 h to 14 day on the injured cornea. It was found that a decrease in MMP-9 expression on day 14 from immunohistochemical staining. Cryotherapy can reduce MMP-9 expression on the 3rd to 14th days of observation after giving therapy to the lesion. Cryotherapy had effect of reducing the infiltration of macrophages that secreted MMP-9, resulting in decreased expression of MMP-9. This is consistent with the conducted research, the expression of MMP-9 decreased in cryotherapy group.

In this study result, cryotherapy had lower TGF-β1 expression than the control group and combined therapy towards cryotherapy and natamycin. There were no statistically significant differences between the control group, natamycin therapy, cryotherapy, and the combination between cryotherapy and natamycin, as seen through IHC staining. It can be due to large data distribution and give the results are not significantly different. The extensive distribution of the data in this study could be due to the immunohistochemical examination carried out once on the 9th day after the inoculation of the Aspergillus flavus fungi. The type of rat strain in this study that is different from previous studies can affect the immune system, which can also be a contributing factor no significant differences, even though this study has followed the procedure from...
the beginning before the animal model of corneal ulcer to the procedure on immunohistochemical staining. It has minimized the possibility that the causes no significant. Infiltration of inflammatory cells varied groups had invaded into anterior chambers of the eye to the lens.

In contrast, there was no observation and counting of the number of cells expressing TGF-β1 apart from the corneal stromal keratocytes in this study. It could lead to no significant difference in the four groups, but in this study, it was found that the expression of TGF-β1 increased in the control group. In contrast, in the cryotherapy group showed a decrease in TGF-β1 expression, which was in accordance with previous studies showed an increased expression of TGF-β1 in the stroma on day 1 and 3 after corneal injury. Another research finding, a mouse model of corneal ulcers, increased the expression of TGF-β1 up to day 14.(6,15) Another study, cryotherapy reduced macrophage infiltration in the injured area, thereby inhibiting and decreased TGF-β1 expression on the 3rd day after administration of cryotherapy.(16)

Natamycin triggers the secretion of IL-1β induces the release of potassium from cells to activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome. Secretion of interleukin (IL)-1β will cause secondary effects after IL-1R activation so that natamycin has the potential to trigger natural immunity. Expression of NLRP3 increases the response to TGF-β1 expression and is associated with MMP-9 expression. MMP-9 is a TGF-β induced gene that plays an essential role in extracellular matrix accumulation and fibrosis. NLRP3 triggers the secretion of IL-1β to stimulate TGF-β1 to induce MMP-9 expression. NLRP3 resides in macrophages that play a role in regulating IL-1β, which contributes to injury and fibrosis formation.(17-20)

Based on the presentation of keratocyte cells expressing TGF-β1 and MMP-9, it was found that cryotherapy was lower than the combination therapy between cryotherapy and natamycin. This study is following the new findings in previous studies regarding the effect of natamycin on the NLR pathway as part of the PRR receptor, which activates the NLRP3 inflammasome so that it can trigger the secretion of IL-1β which affects the increased release of MMP-9 and increased TGF-β1 expression. Therefore, it could allow increasing the expression of TGF-β1 and MMP-9 in combination therapy between cryotherapy and natamycin in this study.(17,19)

Further research can be carried out in the wound healing remodeling phase with observation time (7 day, 14 day, 21 day) and examine other corneal healing parameters in fibrosis factor, collagen structure, and tear examination.

Conclusion

Applying cryotherapy as an alternative to corneal wound healing in the eyes of rats resulted in lower MMP-9 expression, compared to natamycin and combination therapy, but there was no significant difference. The use of cryotherapy as an option for corneal healing in rat eyes resulted in lower TGF-β1 expression than combination therapy but had the same expression as natamycin. Indeed, there were no significant differences between the four groups. In conclusion, cryotherapy can reduce the expression of TGF-β1, but statistically, there is no significant difference in the expression of TGF-β1 or MMP-9.

Authors Contribution

DP, HDS, and IZ designed the experiments and prepared the manuscript. DP and CN participated and performed the experiments, while NK performed the biomolecular analysis. DP and HBN conducted the statistical analysis after the data obtained.

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