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Original Article

Expressions of Matrix Metalloproteinase-3 and Tissue Inhibitor Metalloproteinase-1 in Corneal Tissue Post Alkali Burn Treated with Topical Medroxyprogesterone Acetate and Doxycycline

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ABSTRACT

Purpose: This study aims to investigate the effects of topical Medroxyprogesterone acetate (MPA) and Doxycycline in inhibiting the expression of MMP-3 and TIMP-1 in ocular alkali burn models in animals. **Methods:** A total of 18 New Zealand Rabbits were divided into 3 groups based on their post-alkali-burn treatment: PBS (G1/ control group), topical Doxycycline 1mg/ml (G2), and topical MPA 1% (G3). Alkali burn models were made by exposing 1N NaOH solution to the central cornea for 30 seconds. MMP-3 and TIMP-1 expression were evaluated using immunohistochemistry after 14 days of treatment. **Results:** Statistically significant differences in the mean MMP-3 expression were found between the three groups (p=0.010). There was a significant difference in MMP-3 expression between the control group with MPA (p=0.017) and Doxycycline (p=0.028) but was not found between the MPA and Doxycycline groups (p=1,000). The mean differences in TIMP-1 expression between the three treatment groups were statistically significant (p=0.005), with a significant difference between the control group with Doxycycline (p=0.022) and MPA (p=0.007). There was no significant difference in TIMP-1 expression between the Doxycycline and MPA groups (P=1,000). **Conclusion**: This study indicated that topical administration of Doxycycline or MPA in ocular alkali burn reduces the expression of MMP-3 and TIMP-1.
Key words: Corneal alkali burn, Medroxyprogesterone acetate, Doxycycline.

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INTRODUCTION

Chemical ocular trauma is an ophthalmology emergency. It is classified based on the causal agent, alkali or acidic. Ocular alkali burn constitutes 22% of all ocular trauma and is more prevalent in men, groups age of 20-40 years old and often happen in workplace. Ocular alkali burn often induces more severe damage due to fatty acid saponification on the cell membrane, leading to conjunctivae and corneal cell injury. Alkali penetration stimulates massive keratocyte apoptosis, followed by inflammation with proteolytic enzymes and cytokine. The severity degree (Roper-Hall classification) can be determined based on the extend of the damage, starting from conjunctiva, limbus, and cornea. If left untreated, progressive damage may threaten vision.[1-7]

Wound healing takes place right after the trauma, starting from homeostasis, inflammation, proliferation, and remodeling phase in the cornea. During the inflammation phase, proinflammation cytokine production, such as IL-1, IL-6, and TNF-α, induces TGF- β1 and cell differentiation, from keratocytes to fibroblasts. This process induces matrix metalloproteinase (MMP), including MMP-1, MMP-3, and MMP-9, which take part in the remodeling phase of the stroma. In comparison to other type of MMP, MMP-3 has the most extensive biological activity on extracellular matrix (ECM), type I, IV, and IX of collagen, laminin, preteoglican, and fibronectin. It also induces tissue inhibitors metalloproteinases (TIMP) as MMP inhibitor. TIMP-1 was found to be the most increased, compared to TIMP-2, -3 and 4, during corneal inflammation. The balance between MMP and TIMP is important in ensuring the stability of degradation and matrix deposition.[8-11]

Prompt treatment is required in ocular alkali burn.[12] The goal of the treatment is to increase epithelial integrity and stroma stability, reducing delayed inflammation, as well as to prevent complication. Irrigation should be done immediately to eliminate the causal agent, followed by medication such as antibiotics, steroids, ascorbic acid, amnion membrane transplant, or keratoplasty.[5,13] Doxycycline and Medroxyprogesterone acetate (MPA) had been studied in ocular alkali burn treatment.[14] Doxycycline is a broad-spectrum long-acting tetracycline antibiotic. Doxycycline might protect the cornea by countering medium to severe post-alkali burn collagenolytic degeneration. It also presented with MMP (MMP inhibitor/ MMPI) and neovascularization inhibition effect. Compared to its antibacterial effect, several studies reported that MMPI in tetracycline has a more therapeutic effect on corneal ulcer [10,11] MPA is a progesterone derivate which inhibits IL-1β synthesis that induces collagen degeneration by corneal fibroblast. This effect is related to MMP-1, MMP-2, MMP-3, and MMP-9 expression or activation reduction. MPA is a derivate of 17-α hydroxyprogesterone. MPA has affinity to glucocorticoid receptors, thus has anti-inflammation property. Due to its different pharmacology, it has lower antiinflammation as well as suppression effect compared to corticosteroid.

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Long-term MPA administration has minimal effect on collagen production and wound healing inhibition. Several reports found that ulcers and perforation in rabbits with alkali burn lessen by topical or parenteral MPA administration. MPA was found to have additional effects, including anti-inflammation and less complication compared to steroids.[10,11,15-18]

On one hand, ocular alkali burn is notorious for its vision prognosis. On the other hand, the molecular effect of ocular alkali burn treatment of choice is limited. MPA and Doxycycline might become either a great alternative or complementing treatment.[14] However, there has been not much research that studies MPA biomolecular mechanism, especially comparing its effect to topical Doxycycline. Therefore, this study aims to know whether topical Doxycycline and MPA administration may inhibit MMP-3 and TIMP-1 expression.[19]

MATERIALS AND METHODS

This true experimental with a randomized control group post-test-only design was completed on New Zealand male rabbit (*Oryctolagus cuniculus*) to compare the therapy response of topical Medroxyprogesterone acetate (MPA) with Doxycycline on the post-NaOH-exposure cornea. The total samples were 18 rabbits, which were divided into 3 groups through simple random sampling.

Research samples

The experimental subject was healthy adult male New Zealand rabbits (*Oryctolagus cuniculus*), which were 3 – 4 months of age, and weighing 2.5 – 3.5 kg. Subjects went through a one-week adaptation and were under competent veterinarian observation. The exclusion criteria were if the subject had new corneal injuries during the experiment or had been used in another experiment. Dropout criteria were if the subject was sick or died during the research. The selected research samples were then divided into three groups. After being given NaOH exposure, each group was followed by different topical treatments. Group 1 (G1) as control group was given phosphate-buffered saline (PBS); Group 2 (G2) was given topical Doxycycline; and Group 3 (G3) was given topical MPA.

Procedures

All animal procedures were carried out in accordance with The Animal Care and Use Committee of Airlangga University of Veterinary Faculty (Ethical Clearance No: 2.KEH.003.01.2023). At the beginning of the experiment, anterior segment examination and fluorescein test using a handheld slitlamp were completed on each tested animal.

The research subjects were divided into three groups (G1-G3). Before exposing the cornea with alkali, intramuscular Xylazine hydrochloride (5mg/kg) followed by intramuscular Ketamine Hydrochloride 5% (35mg/kg) was done on every subject. Then, local anesthesia using topical *Tetracaine hydrochloride* 0.5% was given. Every subject central cornea then was placed in a 8-mm diameter sterile filter, which had been soaked in NaOH for 1-2 minutes, for 30 seconds on day 0. After filter removal, ocular surfaces were irrigated using 20 ml of normal saline for 30 seconds, followed by pH measurement. One drop of *Levofloxacin* 1% every 6 hours was administered to reduce the risk of secondary infection. Elizabeth collar was applied to test subjects to prevent scratching. G1 was then given topical PBS, meanwhile, G2 were given topical Doxycycline (Doxycycline hyclate, Interbat, Sidoarjo, Indonesia) 1mg/mL, made by dissolving Doxycycline powder with PBS. G3 were given topical MPA (Triclofem®) 1%, made by diluting 1mL of MPA 150mg/mL into 15mL PBS.

On day 14, every subject went through ocular enucleation followed by corneal slicing for histopathological examination. Corneal tissues were preserved in *neutral buffer formalin* 10% solution for 24 hours. Then, the tissues were washed, dehydrated with ethanol, cleared with xylol, and impregnated with liquid paraffin, and then embedded in paraffin. The paraffin block was then sliced using a microtome at 4 µm thickness. To examine the expression of MMP-3 dan TIMP-1, immunobiological staining was done using MMP-3 and TIMP-1 antibodies on a sliced paraffin block. Stained tissues were observed under a light microscope with 400x magnification.

Statistical analysis

Data normality test was done using *Shapiro-Wilk* test. Normal distributed and homogenous data variation was then analyzed using *One-way* ANOVA test and followed by *post hoc Bonferroni* test. Normally distributed data with heterogenous variation will be tested with *One-way* ANOVA *Welch* test and followed by *post hoc Games-Howell* test. Non-normal distributed data would be analyzed using *Kruskal-Wallis* test then followed by Mann-Whitney test. The p-value <0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics 26 software.

RESULTS

Corneal alkali burn model

Alkali burn was conducted in the three groups with NaOH 1N exposure for 30 seconds on the right eye, which was then washed using saline solution for 30 seconds until the ocular surface reached normal pH. Ocular alkali burn grade III-IV Ropper-Hall was evident in the research subjects (Figure 1). To reduce the effect of the environment on the stability and potency of the drug, PBS, MPA, and Doxycycline were prepared every morning for 14 days.

MMP-3 expression on corneal tissue

Immunohistochemistry staining using MMP-3 polyclonal antibody is presented in Figure 1. The results of the immunohistochemistry of MMP-3 expression mean were 7.83±2.71 in the control group, 3.83±2.04 on G2, and 3.5±2.17 in G3 (Table 1). The normality test using the Shapiro-Wilk test and the Levene homogeneity test both resulted in p>0.05. These indicated that data were normally distributed and had homogenous data variants. Hence, the hypothesis test used was one-way ANOVA and post hoc Bonferroni. The result showed that there were statistically significant differences in MMP-3 expression mean on G1-G3 (p = 0.010). On the post hoc Bonferroni test, statistically significant differences were found on G1 and G2 (p = 0.028), as well as on G1 and G3 (p = 0.017). However, there was no significant difference found between G2 and G3 (p = 1,000).

Table 1: Comparison of MMP-3 expression between groups.

Group	n	MMP-3 expression			P value
		Mean ±s.b.	Min	Max	
Control (G1)	6	7.83 ± 2.71^{a}	4	12	
Topical Doxycycline (G2)	6	3.83 ± 2.04^{b}	1	6	0.010*
Topical MPA (G3)	6	3.50 ± 2.17^{b}	1	6	

*p<0.05 = significant
Different ^{ab} *Superscript* indicate differences between groups

Table 2: Comparison of TIMP-1 expression between groups.

Group	n	TIMP-1 expression			P value
		Mean ±s.b.	Min	Max	
Control	6	7.67 ± 3.20^{a}	2	12	
Topical Doxycycline	6	3.33 ± 1.75^{b}	1	6	0.005*
Topical MPA	6	2.50 ± 2.07^{b}	1	6	

*p<0.05 = significant
Different ^{ab} *Superscript* indicate differences between groups

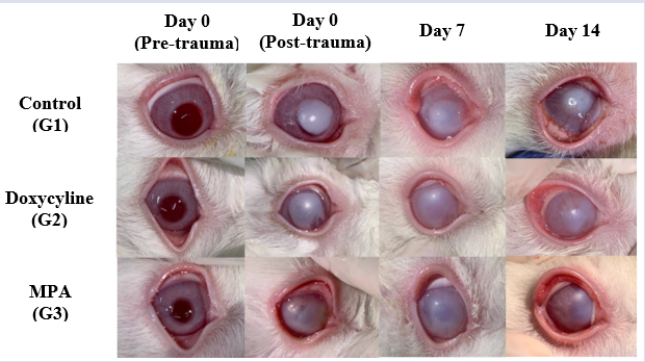

Figure 1: Clinical sample on the three groups (control, topical Doxycycline, and topical MPA) on day 0 (before and after alkali burn), day 7, and day 14 after treatment

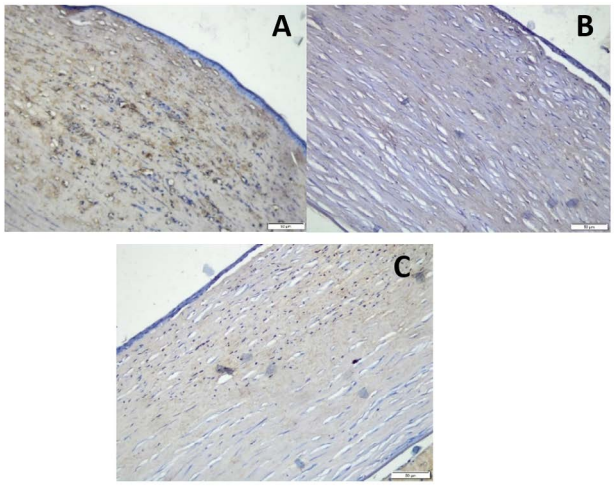

Figure 2: MMP-3 antibody immunohistochemistry result with 200x magnification showing strong intensity on G1 (A), medium intensity on G2 (B) and weak intensity on G3 (C)

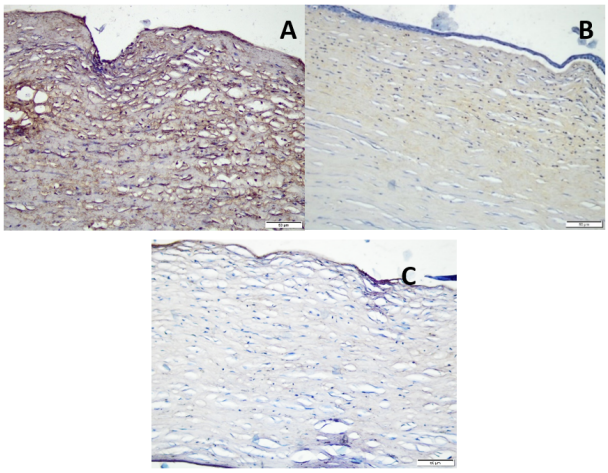

Figure 3: TIMP-1 antibody immunohistochemistry result with 200x magnification showing strong intensity on G1 (A), medium intensity on G2 (B) and weak intensity on G3 (C)

TIMP-1 expression on corneal tissue

Immunohistochemistry staining using TIMP-1 polyclonal antibody is presented in Figure 2. The results of the immunohistochemistry TIMP-1 expression mean on G1, G2, and G3 were 7.6 ± 3.20, 3.3 ± 1.75 and 2.5 ± 2.07, respectively (Table 2). P>0.05 was found both on the Shapiro-Wilk test and Levene homogeneity test, indicating normal distribution and homogenous data variants. One-way ANOVA and post hoc Bonferroni were done afterward. Statistically significant differences in TIMP-1 expression mean were found in every group (p = 0.005). The Post hoc Bonferroni test showed statistically significant differences between G1 and G2 (p = 0.022), as well as on G1 and G3 (p = 0.007). There was no difference between G2 and G3 (p = 1,000).

DISCUSSION

Cornea is a transparent avascular tissue which function as protection from infection in the eye. Cornea consists of cellular (epithelial, keratocyte and endothelial cell) and acellular (collagen and glycosaminoglycan) component. Along with tear film, cornea contributes to two thirds vision refraction Cornea has gradually thickened of thickness from the central to the peripheral side.[1,20,21] Sample used in this experiment were New Zealand rabbit (*Oryctolagus cuniculus*). Rabbit has widely used in corneal study due to its similarity to human cornea, such as diameter, wound healing mechanism, thickness, and composition of the cornea. Several studies mentioned that rabbit cornea has similar corneal density, central thickness and age-related corneal diameter reduction to human.[22-24] This study used only male rabbit to avoid hormonal bias. It is supported by previous research by Zhou *et al.*, which indicate that woman hormone such as Progesterone might affect the collagen content in corneal stroma under physiological or pathological condition.[16]

Ocular alkali burn rapid and extensive damage is related to its pathophysiology.[12] Alkali agent has hydroxyl molecule which can cause lipid saponification. When there is damage in the membrane cell function, cell death occurs and leads to more efficient penetration of the alkali agent. It may penetrate deep enough to the collagen fibril that prone to enzymatic degradation.[5,25] During necrosis liquification, inflammation response induces the release of proteolytic enzymes that may cause further damage.[6,7]

The increase expression of MMP, particularly significant in MMP-1, MMP-3, MMP-8, and MMP-9, occurs during wound healing phase post severe inflammation due to ocular alkali burn. MMP function in ECM and basal membrane component degradation. It also has role in ECM remodeling, cytokine activation and angiogenesis regulation. MMP-3 is known to have the most extensive biology activity in the eye tissue. MMP-3 is secreted only by the fibroblast cell, which are known to increase during corneal injury.[26-28] This study showed significant differences between the three groups on MMP-3 expression using the IRS score. MMP-3 expression was found to be lower on topical Doxycycline and the MPA group. Doxycycline is widely known as an MMP inhibitor, including MMP-3. Topical Doxycycline 0.1% reduced MMP-1, MMP-3, MMP-9, MMP-13 dan TIMP-1 in Bian *et al.* study. Doxycycline functioned as MMP non-competitive inhibitor by interacting with Zinc (Zn) and Calcium (Ca) for stability to occur. This chelation ability with metal ions by Doxycycline allows collagenase reduction.[29] Similarly, MPA impact on MMP-3 was supported by previous studies. Zhou *et al.*, who examine MMP-3 expression on corneal fibroblast cells given MPA with different concentrations, indicated that MPA significantly inhibits MMP expression, including MMP-1 MMP-2, MMP-3, and MMP-9 through IL-1β pathway inhibition.[30] MMP-3 was also known to be pro-MMP-9 which converts inactive MMP-9 into its active form. MPA was found to decrease MMP-9 expression, including MMP-3 as its activator. MPA ability on MMP complex inhibition resulted in MMP extracellular matrix

(EMC) degradation effect reduced.[17] Compared to topical steroids, the main therapy in ocular alkali burn, MPA presented with lower side effects. Topical dexamethasone was found to increase MMP-8 expression which might increase immunoreactivity on inflammation. This might induce excessive inflammation and result in corneal ulcer complications in long-term usage.[29] Based on these researches, MPA might potentially become the therapy for reducing corneal inflammation. Though Doxycycline and MPA have similar effects on reducing MMP, the mechanisms are different.[14] This might explain the insignificant differences between the two groups as ocular alkali burn therapy.[31]

TIMP-1 is a natural MMP inhibitor that function as MMP-3 antagonist. TIMP-1 is secreted by majority cells which inhibit MMP, except MMP-14, MMP-16, MMP-18, MMP-19, MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP. The balance of TIMP and MMP is important to ECM integrity. At the beginning, TIMP binds to MMP hemopexin domain, followed by catalytic domain binding which then eliminate Zinc from the active site. This result in MMP enzymatic activity disturbance.[32-34] In this experiment, TIMP-1 was lower in the Doxycycline and MPA group. Despite being well-known as an MMP inhibitor, not much is known about how Doxycycline affects the expression of TIMP. Mata *et al*. examined Doxycycline in abdominal aortic aneurysm treatment and reported a reduction in TIMP-1 and TIMP-2 expression alongside MMP-2 and MMP-9 expression reduction. Doxycycline is a strong MMP inhibitor, which explains TIMP reduction through an MMP-dependent mechanism. There have been studies on the MPA effect on TIMP-1 in ocular alkali burn. TIMP-1 expression was reported to decrease in corneal fibroblast cell which was given MPA in various concentration. TIMP-1 is directly dependent on MMP expression to inhibit MMP activity. MPA impedes the IL-1β pathway, therefore affecting MMP and TIMP expression which play a role in ECM degeneration, including collagen that contributes to the wound healing process. MMP expression reduction is directly correlated with TIMP expression reduction.[30] In this mechanism, MMP expression lessens by the time TIMP is reduced.[35]

Contrary, Bian *et al*. reported that topical Doxycycline 0.1% on ocular alkali burn did not reduce TIMP-1 expression.[12,30,36] This might be because TIMP-1 works in two mechanisms: MMP-dependent mechanism and the MMP-independent mechanism. In MMP-dependent mechanism, TIMP reversibly blocks MMP activity with a stoichiometric ratio of 1:1, therefore reducing ECM degradation. In MMP-independent mechanism, TIMP modulates cell growth, proliferation, migration, and angiogenesis by binding receptors and inducing specific signaling pathways. Both TIMP mechanisms suppress microvascular endothelial cell migration.[37] These different mechanisms might explain why MMP did not always decrease with TIMP. An aligned MMP-dependent mechanism might explain the reason why TIMP-1 decreased along with the decrease of MMP-3 in this study.

This research has some limitations. The research was done at one time and was not conducted in a serial manner at different times based on each corneal healing phase. Further research should be done with other methods such as Enzyme-linked Immunosorbent Assay (ELISA) to measure the MMP-3 and TIMP-1 expression to be able to compare the ratio of MMP/TIMP. Experiments using different dosages of Doxycycline and MPA might be used to evaluate the therapeutic and toxicity effect of the drug on the anterior segment of the eyes. Different times of Doxycycline and MPA might be able to understand their potencies as ocular alkali burn therapy as well as the MMP-3 and TIMP-1 expression. Other MMP or TIMP variables might be used to evaluate the effect of MPA and Doxycycline on the same matter.

CONCLUSION

MMP-3 and TIMP-1 expressions were found to be lower in the Doxycycline and MPA groups compared to the control group. MPA and Doxycycline might help promoting corneal healing and reducing the progression of corneal fibrosis. This indicates that both MPA and Doxycycline might become adjuvant treatments for ocular alkali burn.

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CONFLICTS OF INTEREST

The author declared that there are no conflicts of interest.

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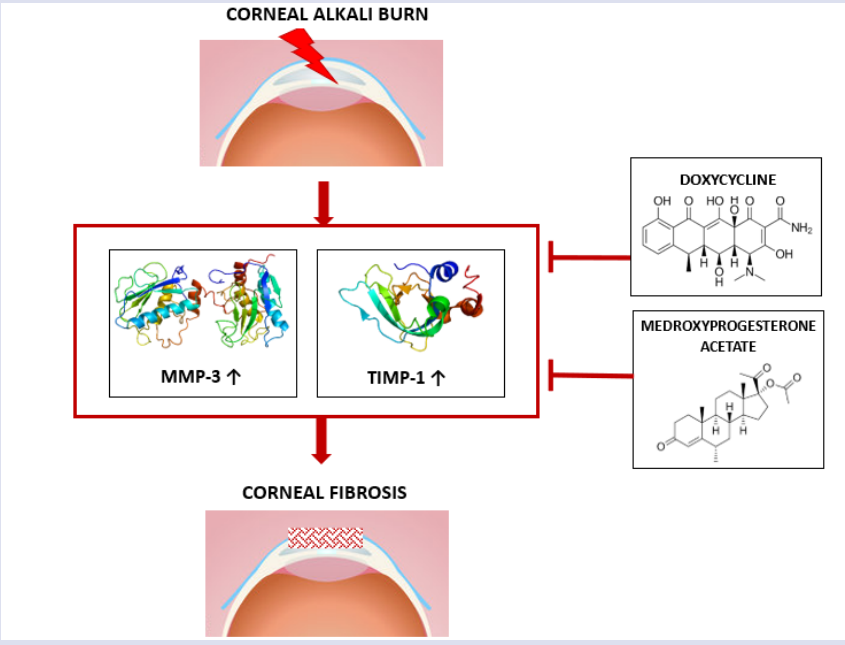
GRAPHICAL ABSTRACT



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