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Research Article

Comparison of Asiatic Acid and Dexamethasone Effect on Interleukin-4 Expression and Eosinophile Cell Count Following Strabismus Surgery: An Experimental Study in New Zealand Rabbit

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ABSTRACT

Introduction: Postoperative strabismus inflammation can lead to fibrotic tissue formation. Dexamethasone, while generally effective as an anti-inflammatory post-surgery medication, can increase IOP and blood sugar levels. Research on asiatic acid suggests its potential as an anti-inflammatory and anti-fibrotic agent. **Methods:** Superior rectus recession was conducted on 20 rabbits, which were divided into five groups based on the subconjunctival injection substance: aquadest, dexamethasone, asiatic acid at a concentration of 0.4 mg/0.5 mL, 0.8 mg/0.5 mL, and 1.6 mg/0.5 mL. After three days, exenteration was performed, and an immunohistochemical examination was performed to assess interleukin-4 expression. Hematoxylin and eosin staining was performed to assess eosinophile cell count. SPSS 26.0 facilitated the data analysis using the Kruskal-Wallis and Wilcoxon Mann-Whitney tests. $P<0.05$ was considered significant statistically. **Results:** This study showed that interleukin-4 expression in the asiatic acid 0.4 mg/0.5 mL group was significantly decreased compared to the aquadest group ($P = 0.029$) and dexamethasone group ($P = 0.029$). Higher-dose groups did not exhibit a significant decrease. Dexamethasone also did not exhibit a significant decrease compare to aquadest. There was no significant reduction of eosinophile cell count among all groups. **Conclusions:** This study highlighted the potential of asiatic acid, particularly at the concentration of 0.4 mg/0.5 mL, in reducing the inflammatory response, specifically interleukin-4 expression, after strabismus surgery in New Zealand rabbits.

Keywords: Interleukin-4, Eosinophil, Strabismus, Asiatic acid, Dexamethasone.

INTRODUCTION

Management in cases of strabismus needs to be carried out to restore binocular vision function in sufferers. The prevalence of strabismus worldwide is 1.93%. Most cases of strabismus require surgical treatment. Strabismus surgery itself is a reconstructive procedure, not a cosmetic one, because this surgical procedure has functional and psychological benefits for the patient. However, strabismus can recur due to the formation of scar tissue that attaches tissues that should not be attached, including the extraocular muscles, tenons, conjunctiva, and sclera after surgery. A report from Repka et al. (2018) stated that the incidence of reoperation in strabismus patients in the United States reached 6.72%.[1-4]

Scar tissue forms during the chronic phase of wound healing. This process begins in the acute phase, where blood cells infiltrate, which produce cytokines that trigger the fibrosis process, resulting in scar tissue. The blood cells involved include polymorphonuclear cells (eosinophils, neutrophils) and mononuclear cells, while several cytokine components involved are IL-1, Tumor Necrosis Factor-α (TNF-α), and type 2 cytokines (IL-4, IL-13). Strabismus surgery hopes to limit the wound healing process so that fibrous tissue forms as a neat, thin line that connects the tendon to the sclera at the insertion point. Excessive inflammation will cause excessive fibrosis due to excessive deposition of extracellular matrix components, including collagen, which will trigger unwanted adhesions and cause eyeball movement problems that affect surgery results.[1,5]

Corticosteroid is a potent and effective anti-inflammatory. One of the most potent corticosteroids is dexamethasone. However, dexamethasone has side effects; susceptible individuals may experience an increase in intraocular pressure (IOP) after dexamethasone administration, even up to 22 mmHg, from administering dexamethasone topically. Subconjunctival administration of dexamethasone can also increase blood sugar levels via systemic absorption. On the other hand, asiatic acid, which is a pentacyclic triterpenoid derivative from *Centella asiatica*, has anti-inflammatory capabilities. Another study using asiatic acid was carried out on corneal, kidney, and liver epithelial cells, where the results showed a reduction in inflammatory cytokines. Research by Kurniasih et al. (2021) shows that administering 0.4 mg/0.5 mL subconjunctivally after trabeculectomy surgery can reduce fibroblasts. In addition, asiatic acid has been studied as a potential glaucoma therapy because it has a neuroprotective effect on retinal ganglion cells.[6-12]

This study aimed to compare the effectiveness of dexamethasone and asiatic acid in reducing inflammation after strabismus surgery caused by type 2 immunity. Specifically, this study examined the inflammation using IL-4 expression and eosinophil

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cell count in muscle, conjunctiva, and tenon tissue. Theoretically, if the inflammation is reduced, the fibrosis will also be reduced. If asiatic acid is effective in reducing inflammation, then asiatic asid can be used as an alternative to prevent fibrosis after strabismus surgery.

MATERIALS AND METHODS

Asiatic acid preparation

Asiatic acid powder with a purity level of >97% (Tokyo Chemical Industry Co., Ltd.) was weighed at 48 mg, then dissolved in dimethyl sulfoxide (DMSO) and phosphate buffer saline (PBS) according to the procedure for making asiatic acid preparations in the research of Yang et al. (2018), in this study a ratio of 1:4 was used (3 mL DMSO and 12 mL PBS). The dissolution process uses ultrasonic waves for 30 minutes as a stirring medium. The solution was then divided into 3, each with 5 mL, and then the two solutions were diluted with PBS (5 mL and 15 mL each). The final asiatic acid concentration of each solution was 0.4, 0.8, and 1.6 mg/0.5 mL, with a pH of 6.9. The process ends with sterilization of the solution using ultraviolet light for 30 minutes.[10]

Animal model preparation

This study used male New Zealand white rabbits (*Oryctolagus cuniculus*), aged 4-10 months old, weighing around 2.0-3.0 kg, and with healthy eyes and body conditions. Twenty experimental animals that met the criteria were randomized into five groups, each consisting of four experimental animals. The experimental animals underwent acclimatization for seven days. After acclimatization, the experimental animals underwent superior rectus recess surgery on the right eye. The new muscle insertion was measured using calipers as far as 2 mm from the initial insertion. The procedure ended with the administration of topical antibiotic drops of levofloxacin hemihydrate 0.5% (Neo Levo; Rohto, Indonesia) and 0.5 mL subconjunctival injection using substance that was assigned to each group of experimental animals (aquadest, dexamethasone 2.5 mg/0.5 mL, asiatic acid 0.4 mg/0.5 mL, 0.8 mg/0.5 mL, 1.6 mg/0.5 mL). None of the animals were infected or died during the study. All experimental animals underwent exenteration three days after superior rectus recess surgery. One operator carried out each surgical procedure. The exenterated eyeballs were cut to make histologic preparations according to the research procedure of Bani et al. in the form of a sagittal cut, with a width of 4 mm and a length of 6 mm (2 mm anterior to the new insertion to 4 mm posterior to the new insertion). The preparations were evaluated by an anatomical pathology specialist.[13]

IL-4 expression assessment

IL-4 expression was examined using immunohistochemistry (IHC) staining. Assessment of IL-4 expression was carried out semiquantitatively using the modified Remmele method. Immuno Reactive Score (IRS) is obtained by multiplying the percentage score of the immunoreactive cells with the staining intensity score of the immunoreactive cells. The percentage score of the immunoreactive cells is divided into five categories: score 0 (no positive cells), score 1 (positive cells <10%), score 2 (positive cells 10-50%), score 3 (positive cells 51-80 %), and score 4 (positive cells >80%). The staining intensity score of the immunoreactive cells is divided into four categories: score 0 (no color reaction), score 1 (mild reaction), score 2 (moderate reaction), and score 3 (intense reaction). IRS was observed in five fields of view at 400x magnification; the results were then averaged to obtain IRS data on one sample. The IRS score has a value range of 0-12, then divided into four categories: negative expression (score 0, scale 1), mild expression (score 1-4, scale 2), moderate expression (score 5-9, scale 3), and strongly positive expression (score 10-12, scale 4).[14]

Eosinophil cell count assessment

The eosinophil cell count was examined using hematoxylin and eosin (H&E) staining. The tissue preparations that had been stained were then evaluated using a light microscope at 10x magnification to look for areas with the densest population of inflammatory cells. After obtaining the desired area, the examination continues with 400x magnification to count the number of eosinophils in three adjacent areas. The number of eosinophils from the three areas was averaged and categorized into mild (1-50 cells, scale 1), moderate (51-100 cells, scale 2), severe (101-200 cells, scale 3), and very severe (> 200 cells, scale 4).[15]

Ethical approval

The ethical approval for the feasibility study was granted by the Research Ethics Commission of the Faculty of Veterinary Medicine Animal Care and Use Committee (ACUC) at Universitas Airlangga, Surabaya with the reference number 2.KEH.064.04.2023.

Statistical analysis

IL-4 expression and eosinophil cell count data are presented in scale (ordinal data). Comparative data on IL-4 expression and eosinophil cell count with five interventions were tested using the Kruskal-Wallis test. If significant results are obtained, proceed with the Wilcoxon Mann-Whitney test to determine whether the differences between one group and another are significant. All data were analyzed and presented as median, minimum, and maximum values. $p<0.05$ was considered a significant p value. The software that was used to process all statistical data was SPSS 26.0.

RESULTS

This study examined IL-4 expression and eosinophil cell count in muscle, conjunctiva, and tenon tissue. Conjunctiva and tenon tissue were examined as one part. IL-4 expression and eosinophil cells were not found in muscle tissue. We only performed statistical analysis of IL-4 expression and eosinophil cell count in conjunctiva and tenon tissue.

The IL-4 expression in the conjunctiva and tenon tissue has descriptive data as shown in Table 1, where it was found that the highest median value was in the asiatic acid group 0.8 and 1.6 mg/0.5 mL with a value of 3, while the lowest was obtained in the 0.4 mg/0.5 mL asiatic acid group with a value of 1. The minimum and maximum IL-4 IRS values in the 0.4 mg/0.5 mL asiatic acid group were the same, which is 1, whereas, in the other four groups, the minimum and maximum values are the same, 2 and 3, respectively. Kruskal-Wallis test showed a statistically significant result ($p = 0.02$). The statistical analysis was continued for each group using the Wilcoxon Mann-Whitney test. Significant results were only obtained in the 0.4 mg/0.5 mL asiatic acid group with all other groups, while the other four groups were not significant with each other; details of these are outlined in Table 1.

This study showed that only asiatic acid at a 0.4 mg/0.5 mL dose provided significant result compared with the aquadest in reducing IL-4 expression in the conjunctiva and tenon. There was no dose-dependent relationship because there was no significant decrease when compared with a dose of asiatic acid greater than 0.4 mg/0.5 mL; apart from that, in the 0.8 and 1.6 mg/0.5 mL dose groups also showed that IL-4 expression was greater than the 0.4 mg/0.5 mL dose and was not significant compared to the aquadest. Dexamethasone in this study also showed insignificant result when compared to the aquadest.

The eosinophil cell count was homogeneous in all of the preparations, with a value of 1 from the median value, minimum value, and

Table 1. IRS IL-4 expression scale comparison test.

Group	Median	Min	Max	p value*	p value**
Aquadest	2,50	2	3		II = 0,686 III = 0,029*** IV = 0,686 V = 1
Dexamethasone	2	2	3		I = 0,686 III = 0,029*** IV = 0,343 V = 0,686
Asiatic acid 0,4 mg/0.5 mL	1	1	1	0,02***	I = 0,029*** II = 0,029*** IV = 0,029*** V = 0,029***
Asiatic acid 0,8 mg/0.5 mL	3	2	3		I = 0,686 II = 0,343 III = 0,029*** V = 0,686
Asiatic acid 1,6 mg/0.5 mL	2,50	2	3		I = 1 II = 0,686 III = 0,029*** IV = 0,686

* Kruskal-Wallis test ** Wilcoxon Mann-Whitney test *** Indicates statistically significant
I = Aquadest
II = Dexamethasone
III = Asiatic acid 0,4 mg/0.5 mL
IV = Asiatic acid 0,8 mg/0.5 mL
V = Asiatic acid 1,6 mg/0.5 mL

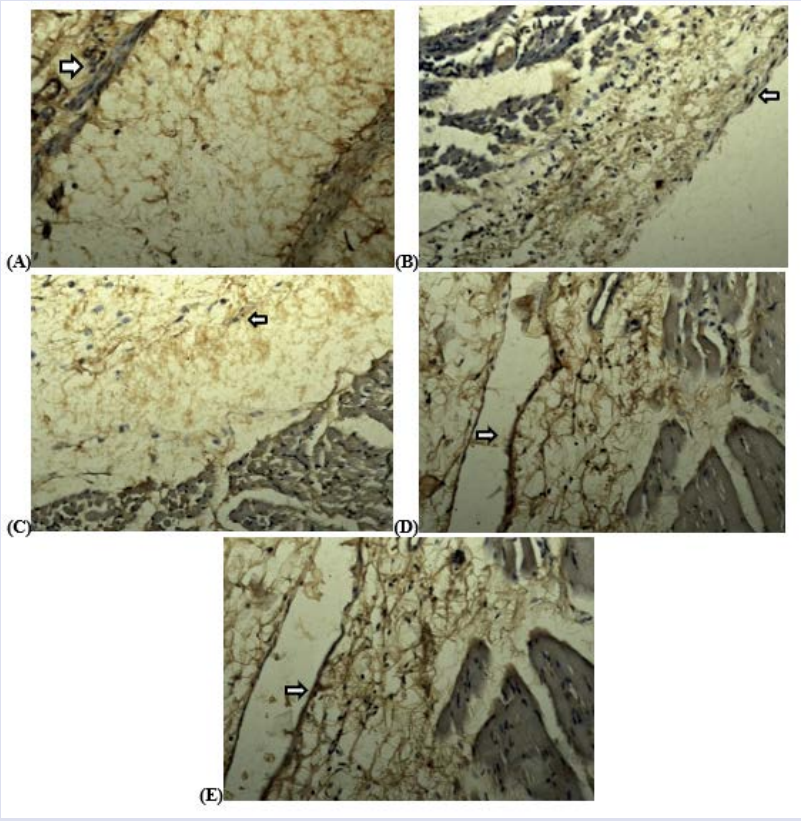

Figure 1: IHC staining results. IL-4 expression appears as a brown color in the cytoplasm of inflammatory cells at 400x magnification as indicated by the white arrow. It appeared that IL-4 expression was only present in the conjunctiva and tenon, whereas in the extraocular muscles there was no IL-4 expression. (A = aquadest; B = dexamethasone; C = asiatic acid 0.4 mg/0.5 mL; D = asiatic acid 0.8 mg/0.5 mL; E = asiatic acid 1.6 mg/0.5 mL).

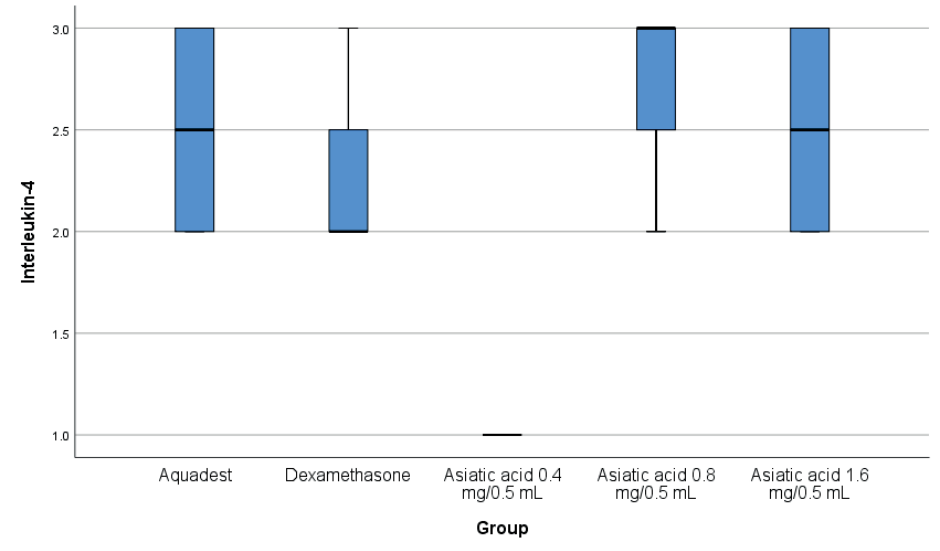

Figure 2: **Boxplot comparison of IL-4 expression.** Asiatic acid 0.4 mg/0.5 mL is the only group that is significantly has lower IL-4 expression compared with the other groups.

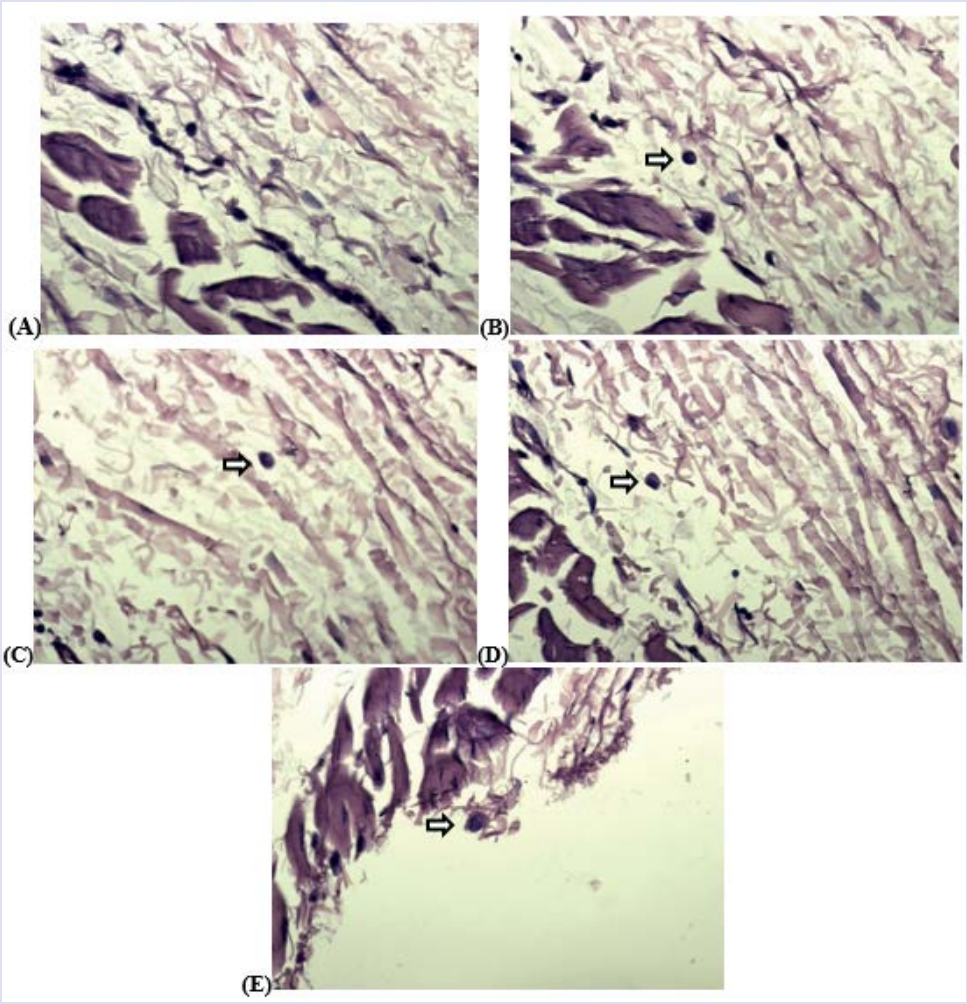

Figure 3: HE staining results. Eosinophil cells appear as cells with bilobulated nuclei at 400x magnification, as indicated by the white arrow. No eosinophil cells were found in all preparations from the aquadest control group. (A = aquadest; B = dexamethasone; C = asiatic acid 0.4 mg/0.5 mL; D = asiatic acid 0.8 mg/0.5 mL; E = asiatic acid 1.6 mg/0.5 mL).

maximum value. Statistical tests were not carried out because the data were homogeneous. Raw data on the number of eosinophil cells before categorization showed a result of 0-2 in conjunctival and tenon tissue. In the aquadest group, no tissue showed eosinophil cells.

DISCUSSION

The wound healing process begins after strabismus surgery. The phases of wound healing include hemostasis, inflammation, proliferation, and remodeling. The inflammatory phase is characterized by signs of inflammation, such as edema and erythema in the wound. This phase aims to create immune protection against microorganisms that invade the wound by involving the humoral and cellular inflammatory systems. The initial inflammatory phase occurs with activation of the complement cascade and neutrophil infiltration from 24-36 hours after injury to prevent infection. Neutrophil cells carry out phagocytosis to destroy bacteria, foreign particles, and damaged tissue.[16-20]

Damaged cells will release damage-associated molecular patterns (DAMPs), which will be recognized by pattern recognition receptors (PRRs) from neutrophils, activating the innate immune response by signaling IkB kinase (IKK) to phosphorylate nuclear factor-kB (NF-κB). NF-κB will release pro-inflammatory cytokines such as IL-1, TNF-α, chemokines, and antimicrobial proteins. In the adaptive immune response, NF-κB will activate B cells and T cells so that these two cells will differentiate and proliferate, where T cells can differentiate into T Helper 1 (Th1), Th2, Th9, and Th17. Th2 will produce IL-4, IL-5, and IL-13 and stimulate mast cells, basophils, and eosinophils. NF-κB is also produced from the phosphatidylinositol 3-kinase (PI3K) signaling cascade, and NF-κB is inhibited by nuclear factor erythroid 2-related factor (Nrf2). Asiatic acid inhibits pro-inflammatory cytokines by reducing activation through the IKK pathway to NF-κB and upregulating Nrf2, which inhibits NF-κB. Dexamethasone inhibits PI3K, resulting in a decrease in NF-κB.21,22,23,24

Damaged tissue after strabismus surgery will give rise to type 2 immunity in that tissue, where type 2 immunity involves IL-4, IL-5, IL-13, and recruitment of eosinophils. As part of type 2 immunity from acute muscle tissue damage, eosinophils play a vital role in muscle regeneration. Eosinophils produce type 2 cytokines, namely IL-4 and IL-13. These cytokines play a role in differentiating T cells into Th2, which also produce IL-4, IL-5, and IL-13 again. The response from IL-4 and IL-13 will cause fibroblast cells to produce collagen, which helps close the wound. This pathway is also called the type 2 fibrosis pathway. In addition, IL-4 will stimulate cells in striated muscle, namely fibrogenic/adipogenic progenitors (FAPs), to produce IL-33 and proliferate, which will then differentiate into fibroblast cells. IL-33 will stimulate group 2 innate lymphoid cells (ILC2s) to produce IL-4, IL-5, and IL-13. IL-5 will recruit eosinophils. Eosinophils become excessive in chronic and highly polarized type 2 immune response conditions. Excessive eosinophils in this condition can cause fibrosis due to persistent and excessive signaling, causing excess extracellular matrix deposition, which causes scarring and fibrosis. Eosinophils in the wound will peak on day 3 and days 2 to 4 for IL-4.[21,25-29]

IL-4 expression in this study showed a significant decrease in the 0.4 mg/0.5 mL asiatic acid group compared to all other treatment groups. Our findings are consistent with the research by Kurniasih et al. (2021), which states that asiatic acid 0.4 mg/0.5 mL can reduce the number of fibroblasts post-trabeculectomy. Dexamethasone did not significantly reduce IL-4 expression when compared with the aquadest. Gaballa et al. (2021) state that subconjunctival dexamethasone injection is less optimal for treating inflammation on the ocular surface compared to topical administration; this is due to the dexamethasone that comes out of the subconjunctiva during administration via the injection route, thus reducing the dexamethasone depot, and according to Fukushima et al. (2001), systemic absorption of dexamethasone through the conjunctival and episclera blood vessels occurs quickly so that it can affect the bioavailability of dexamethasone in the subconjunctiva. This is the possibility why dexamethasone did not affect IL-4 expression in our study.[6,11,12]

Asiatic acid doses of 0.8 mg/0.5 mL and 1.6 mg/0.5 mL did not significantly affect IL-4. This shows that in this study, the effect of asiatic acid on IL-4 was non-dose-dependent. Similar results were obtained in research conducted by Wulan et al. (2014), who examined the effect of *Centella asiatica* on the thickness of the pyramid layer in the CA1 area of the hippocampus in stress-induced Sprague Dawley rats. Yuliani and Linar's (2019) research also shows the non-dose-dependent manner of *Centella asiatica* extract on Caspase 3 expression in a mouse model of dementia. Asiatic acid is a component of *Centella asiatica*, so it is possible that the same non-dose-dependent manner can occur. In contrast, research by Moon et al. (2021) and Xu et al. (2017) show that asiatic acid works in a dose-dependent manner. Differences in results from this study may be caused by variations in drug bioavailability, which can be influenced by differences in the route of administration, drug dosage form, organ, and experimental animal species.[8,31-32]

The number of eosinophil cells in the conjunctiva and Tenon was found to be 0-2 in our study. Our study results do not match the theory of Zeinoun et al. (2009), which states that eosinophils will peak on day 3 post-trauma. The cause of the low number of eosinophil cells in this study is probably due to the number of eosinophil cells not increasing significantly after trauma in the conjunctiva, research from Greiner et al. (1985) who traumatized the conjunctiva of mice by rubbing the upper eyelids and found that eosinophils did not increase significantly within 24 hours of observation, in contrast to macrophages which increased significantly. Ogawa et al. (2020) studied the role of eosinophils in the eye regarding the wound healing process. The mice in their study were injured in the corneal epithelium, and it was found that eosinophils were recruited to the limbus. Our study did not examine the limbal tissue; it is possible that we would have been able to find more eosinophils if we had examined the limbus.[26,33,34]

Eosinophils circulate in the blood, but eosinophils predominantly reside in the tissues. There are several hundred times more eosinophils in epithelial tissue than in peripheral blood. Eotaxin is a chemoattractant produced by activated eosinophils; eotaxin plays a specific role in attracting eosinophils to the tissue. Eotaxin is mainly found in the small and large intestines, where eosinophils are needed to defend against helminth pathogens. Eotaxin expression is increased in autoimmune diseases such as ulcerative colitis and Crohn's disease. This is another possibility of a low number of eosinophils; eotaxin did not increase because strabismus surgery is not an autoimmune condition or a worm infection.[35]

The IL-4 expression and the eosinophil cell count in the extraocular muscles in this study showed homogeneous results; no IL-4 expression and no eosinophil cells were found in the extraocular muscles. An examination of IL-4 in extraocular muscles was carried out by Hiromatsu et al. (2000), who detected the presence of IL-4 in the form of mRNA in extraocular muscle tissue and orbital fat. IL-4 was only detected in one extraocular muscle tissue out of 14 tissues examined. This is the possible reason why our finding showed no IL-4 expression in extraocular muscle tissue.[36]

No studies have observed eosinophils in post-traumatic/post-surgical extraocular muscles. However, there is another study conducted by Ben Artsi et al. (2021), who observed eosinophils in extraocular muscle biopsy tissue in patients with idiopathic orbital myositis and found eosinophils in four of the nine preparations observed in small numbers compared to other inflammatory cells, such as lymphocytes, plasma cells, and histiocytes. Another possibility as to why eosinophils were not found in extraocular muscle tissue in this study is because extraocular

muscle is not an epithelial tissue, and eotaxin is low because in this study, we made a trauma condition caused by surgical procedure, not an autoimmune condition or worm infection, so eosinophils are not recruited.[35,37]

The first limitation of this study is the method of examining IL-4 in muscles using IHC, which did not show IL-4 expression in this study. Future research could use an IL-4 examination method using messenger RNA detection, such as the research of Hiromatsu et al. (2000), which obtains messenger RNA from IL-4 in extraocular muscles. Apart from that, examination of IL-4 using IHC also depends on the location of the tissue section, so a comprehensive picture of IL-4 expression in the tissue cannot be obtained. Future research can carry out examinations using tissue culture and observe it using an image analyzer to obtain comprehensive IL-4 expression. The second limitation of the study was that the tissue preparation section did not include the limbus, so sufficient numbers of eosinophil cells were not found. Future research can examine the tissue until the limbus; it is hoped that eosinophils will be obtained by examining the limbus, as in the Ogawa et al. (2020) research.

CONCLUSION

Our findings showed that asiatic acid 0.4 mg/0.5 mL reduced IL-4 expression in a non-dose-dependant manner. Previous study has shown that asiatic acid 0.4 mg/0.5 mL reduced fibrosis after trabeculectomy surgery. Dexamethasone showed no effect in reducing IL-4 expression. Asiatic acid 0.4 mg/0.5 mL may reduce fibrosis after strabismus surgery. Further research could be conducted in a more extended study period to evaluate the wound healing process thoroughly after strabismus surgery. Further research could examine asiatic acid at a narrower dose range and examine other cytokines related to wound healing. It is hoped that developments from this research will provide comprehensive information on the role of asiatic acid in wound healing after strabismus surgery.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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