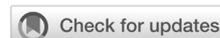


A randomized clinical trial of freeze-dried amniotic membrane transplantation and conjunctival-limbal graft for pterygium excision and evaluation of conjunctival inflammation marker in tears

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pISSN: 0853-1773 • eISSN: 2252-8083
<https://doi.org/10.13181/mji.oa.203818>
Med J Indones. 2020;29:359–65

Received: May 31, 2019

Accepted: September 8, 2020

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This online publication has been corrected. The corrected version first appeared on March 31, 2021.

ABSTRACT

BACKGROUND Minimizing inflammation on the ocular surface after pterygium surgery is essential to prevent recurrence. Currently, limited data are available for the comparison between freeze-dried amniotic membrane (FD-AM) and conjunctival-limbal graft (CLG). Therefore, this study aimed to assess conjunctival inflammation and the interleukin (IL)-6 levels in tears after pterygium excision between both techniques.

METHODS This prospective, single-blinded, randomized clinical trial included 26 eyes of 26 patients with primary or recurrent pterygium and who were indicated for pterygium surgery at Cipto Mangunkusumo Hospital, Indonesia from October 2017 until February 2018. The patients were scheduled for pterygium excision with FD-AM or CLG based on block randomization. Conjunctival inflammation and the level of IL-6 in tears were measured on day-7 and -28 after surgery.

RESULTS The 26 eyes (13 in each group) were subjected to a complete 1-month follow-up period. Day-7 after the surgery, severe inflammation occurred in 4/13 (31%) eyes having FD-AM and 8/13 eyes (62%) having CLG ($p = 0.59$). The IL-6 tear level increased similarly in FD-AM (22.85 pg/ml) and CLG (23.51 pg/ml) in ($p = 0.78$). No severe adverse events occurred.

CONCLUSIONS Pterygium excision using FD-AM yield a milder inflammation than CLG on day-7 and -28 after surgery. The tear IL-6 levels similarly increased on day-7.

KEYWORDS amniotic membrane, interleukin-6, pterygium

Pterygium is a wing-shaped fibrovascular tissue that develops on the cornea.^{1,2} The pathogenesis is not fully understood, and several factors, such as exposure to ultraviolet radiation, dust, and windy environment, are associated with the development of this disease.^{3–5} Several well-known techniques for pterygium surgery are bare sclera, primary closure, conjunctival flap, conjunctival-limbal graft (CLG), and transplantation of amniotic membrane (AM). The greatest challenge of surgery is recurrence, which varies from 2–89% based on the technique used.^{2–4}

Besides the surgical technique, a recurrence is associated with other factors. High-degree pterygium and persistent postoperative inflammation have a high risk of recurrence.² As shown by Kheirkhah et al,⁵ recurrence is high in cases with a persistent inflammation a month after surgery. The inflammation is triggered and enhanced by interleukin (IL)-6, a cytokine that plays a significant role in the process of inflammation by its pluripotent effect. Several types of inflammation cells are activated by IL-6: macrophages, neutrophils, T-lymphocytes, and B-lymphocytes.

Endothelial cells, fibroblasts, and hepatocytes are also influenced by IL-6 and have an association with inflammation.^{6,7}

Based on these facts, controlling inflammation on the ocular surface after pterygium surgery becomes vital to prevent a recurrence.^{2,4} Cha et al⁸ reported that all (100%) their studied cases underwent excision, and those with CLG had a conjunctival inflammation a week after surgery. AM, which exerts an anti-inflammatory effect, is expected to reduce a conjunctival inflammation within a week after surgery. The anti-inflammatory effect occurs through several mechanisms, including suppression of an IL-6.⁹⁻¹¹ Through this anti-inflammatory effect, AM is thought to help in preventing recurrence.

Currently, only several studies have reported conjunctival inflammation after pterygium surgery, and no study has specifically compared the freeze-dried AM (FD-AM) and CLG with regard to the outcomes of conjunctival inflammation. Hence, this study aimed to compare a conjunctival inflammation and IL-6 levels in tears after pterygium excision, along with transplantation of FD-AM and CLG.

METHODS

This prospective, interventional, randomized study included 26 eyes of 26 patients aged >18 years with primary or recurrent pterygium and who were indicated for surgery. The study was conducted at Kirana Cipto Mangunkusumo Hospital, Jakarta, Indonesia, from October 2017 to February 2018. The exclusion criteria were the presence of other diseases of the ocular surface, such as Steven-Johnson syndrome with ocular involvement, limbal stem-cell deficiency, dry eye, corneal ulcer, uveitis, glaucoma, history of eye surgery within the prior 3 months, and a history of hypersensitivity to topical levofloxacin or topical steroid. The study was approved by the Ethics Committee of the Faculty of Medicine Universitas Indonesia (No: 835/UN2.F1/ETIK/2017) and written informed consents were obtained from each patient. The patients were consecutively scheduled for pterygium excision with FD-AM or CLG based on block randomization. A block size of four was determined by the researchers. All possible balanced combinations of assignment within the block were calculated. Blocks were then randomly selected by an independent person (not the researchers) to determine the patients' assignment to the groups.

All surgical procedures were performed by a single experienced surgeon (MS) to ensure a consistency in the extent of tissue removal. Topical anesthesia was administered with xylocaine in all cases. First, the area to be excised was marked. The pterygium head was undermined and removed from the corneal surface by blunt dissection, and the pterygium body, along with the subconjunctival fibrovascular tissue, was excised. The remaining pterygium tissue attached to the cornea was removed carefully using a crescent knife. The tissue (bare scleral) defect was measured. This was followed by the transplantation of FD-AM or CLG. The FD-AM was obtained from Batan Research Tissue Bank and then transplanted over the bare scleral defect with the epithelial basement membrane side facing up. The free edge of the FD-AM was sutured to the episcleral and surrounding conjunctiva using 10-0 nylon. Finally, the area of transplantation was covered with a therapeutic soft contact lens. In CLG, the graft was harvested from the superior area and then transplanted over the defect using 10-0 nylon suture. After surgery, all patients were prescribed topical levofloxacin and topical prednisolone six times daily for 2 weeks and then gradually reduced to four times daily for 2 weeks.

The patients were evaluated on day-1, -7, -14, and -28 after surgery. The sutures were removed 2 weeks after the surgery. During each visit, an uncorrected visual acuity and a conjunctival inflammation were evaluated. Slit-lamp photography was also performed. On day-28, best-corrected visual acuity and astigmatism measurements using automated keratometer were performed by researchers who were blinded to the subject assignment. The presence of conjunctival inflammation was assessed by an ophthalmologist who did not know the subject assignment. The assessment was conducted based on slit-lamp photographs that were taken at day-7 and -28 after surgery. The photographs were graded using previous studies as reference. The grading system is defined by the number of dilated vessels in the bulbar conjunctiva: grade 0 (no vasodilation), grade 1 (mild; portions of vessel vasodilation), grade 2 (moderate; extensive vasodilation), and grade 3 (severe; overall vasodilation).^{5,8} The assessor of the photographs was blinded to the surgical technique used (FD-AM or CLG). If there were any complication or adverse event, which needed strict evaluation (such as infections and slipped grafts) from photograph evaluation, the time of follow-up was extended based on the condition.

Tear samples were collected before surgery, at day-7, and day-28 after surgery. Basal tear samples were collected non-traumatically from the inferior tear meniscus of both eyes by using micropipettes (Eppendorf, Germany). The procedure was performed carefully to avoid touching the corneal and conjunctival surface. There were 25 µl of tear to measure IL-6 levels. The samples were placed in microtubes (Eppendorf) and stored at -70°C until further examination. Enzyme-linked immunosorbent assay was used to measure the IL-6 levels in tears.

The sample size was calculated using two proportion difference formulas, assuming an α value of 5% ($Z\alpha = 1.96$) and β value of 20% ($Z\beta = 0.84$). The proportions of moderate to severe conjunctival inflammation in the CLG and FD-AM group were estimated to be 100%⁸ and 50%,⁵ respectively, a week after surgery. The calculated sample size for each group was 11 with 10% possibility of drop out and the minimum sample size was 24 eyes.

The data were collected in an Excel 2010 spreadsheet (Microsoft, USA) and analyzed using SPSS software version 20.0 (IBM Corp., USA). For numerical variables, the normality test was conducted with Shapiro-Wilk test. The student's t-test or Mann-Whitney test was performed to compare the IL-6 levels between groups. Chi-square or Fisher's exact test was performed to compare conjunctival inflammation between groups. A p -value of <0.05 was considered statistically significant.

RESULTS

All subjects (13 subjects in FD-AM group and 13 subjects in CLG group) participated until the last follow-up examination (study time completed). Table 1 presents the demographic and clinical characteristics of the subjects. The following three recurrent cases were included in this study: one case in the FD-AM group and two cases in the CLG group. No complications or adverse events occurred in the follow-up period of 28 days.

Figure 1 reveals the conjunctival inflammation 7 days after the surgery. Four (31%) subjects in the FD-AM group had a severe inflammation, whereas most subjects (62%) in CLG group had a severe inflammation. Mild inflammation was more common in the FD-AM group compared with the CLG group, although no statistical significance was observed

Table 1. Demographic and clinical characteristics of subjects

Variable	Group	
	FD-AM, n (%) (N = 13)	CLG, n (%) (N = 13)
Age (years), mean (SD)	58.2 (15.8)	57.3 (13.4)
Male sex	9 (69)	6 (46)
Type of pterygium		
Primary	12 (92)	11 (85)
Recurrent	1 (8)	2 (15)
Degree of pterygium		
1	4 (31)	5 (38)
2	6 (46)	4 (31)
3	3 (23)	4 (31)
Presence of lens opacity (cataract)	6 (46)	7 (54)
Initial BCVA (logMAR), median (min-max)	0.20 (1.30-0.00)	0.60 (1.60-0.00)
Duration of surgery (min), mean (SD)	27.0 (4.1)	29.5 (5.6)
IL-6 level in tears (pg/ml), median (min-max)	4.50 (1.82-11.79)	3.33 (1.32-11.91)

FD-AM=freeze-dried amniotic membrane; CLG=conjunctival-limbal graft SD=standard deviation; BCVA=best corrected visual acuity; IL=interleukin

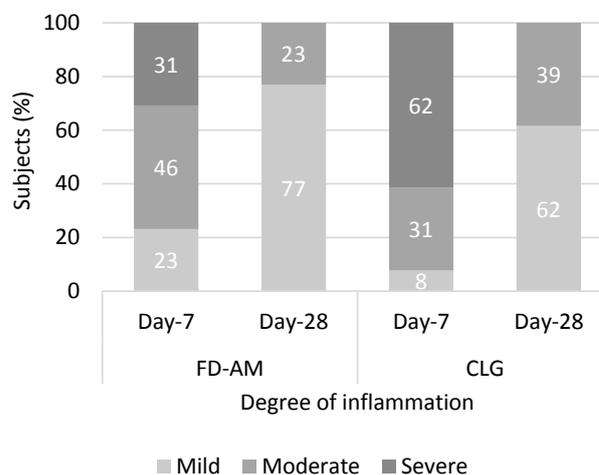


Figure 1. Conjunctival inflammation at day-7 and day-28 after surgery. FD-AM=freeze-dried amniotic membrane; CLG=conjunctival-limbal graft

($p = 0.59$). Figures 2 and 3 show the clinical images of both groups.

Four weeks after surgery, no subject had a severe inflammation in both groups (Figure 1). Most subjects experienced a mild inflammation (77% in the FD-FD-AM group and 62% in the CLG group; $p = 0.67$). All subjects

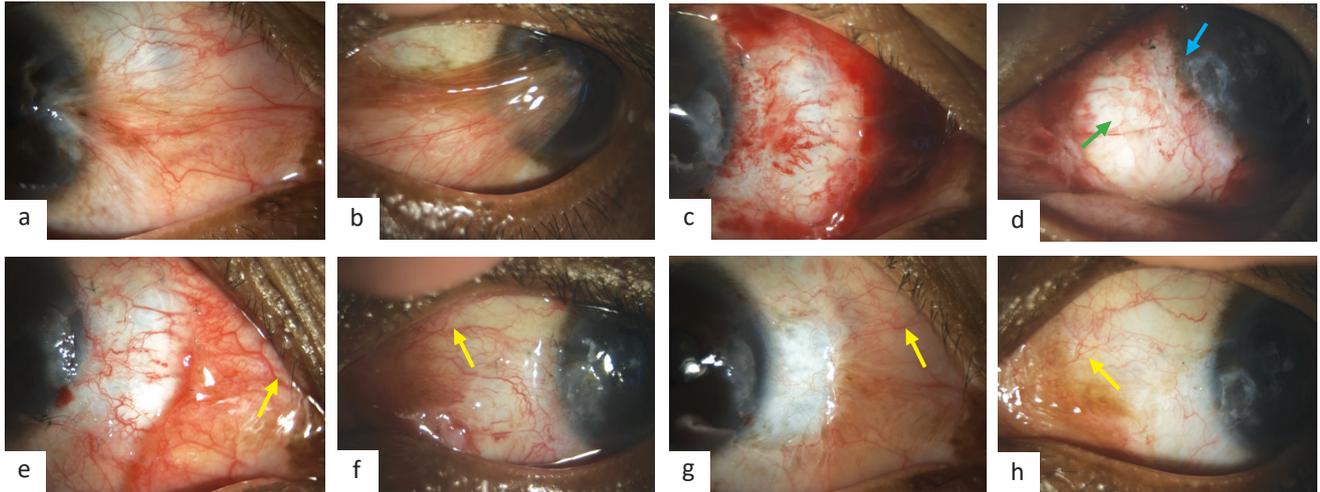


Figure 2. Eyes which underwent excision with freeze-dried amniotic membrane transplantation. Second degree (a) and third degree (b) of primary pterygium; (c) and (d) 1 day after surgery, amnion graft (green arrow) had been transplanted, soft contact lens had been applied (blue arrow). Subconjunctival hemorrhage was present around the graft; (e) and (f) 1 week after surgery, epithelization occurred, and the amnion graft started to dissolve. Moderate inflammation on conjunctiva can be observed, as characterized by dilated vessels (yellow arrow); (g) and (h) 4 weeks after surgery, inflammation around the graft subsided and became mild, characterized by few dilated vessels (yellow arrow)

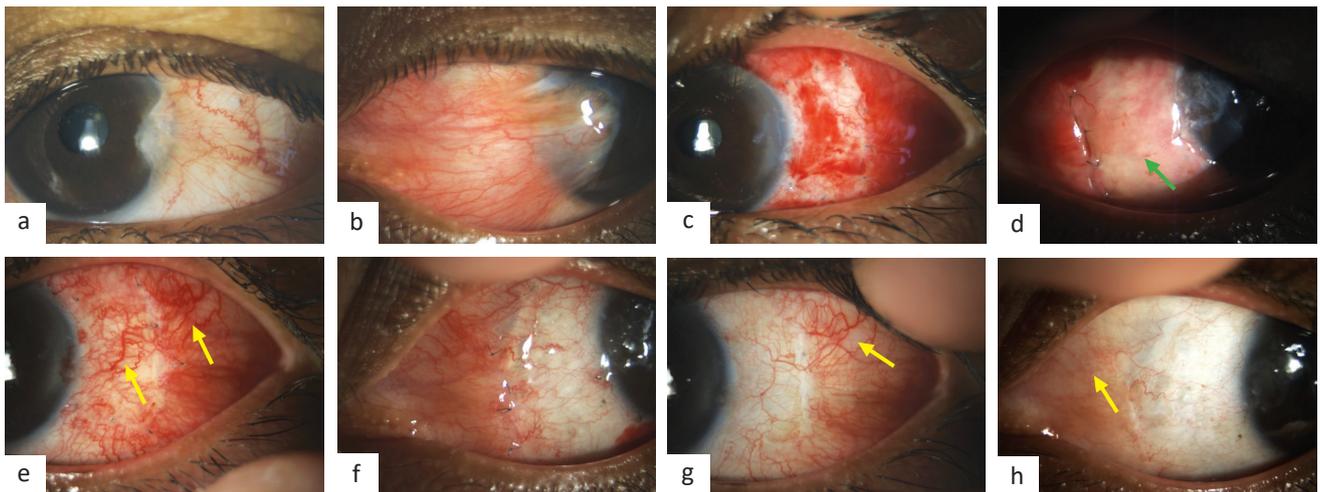


Figure 3. Eyes which underwent excision with conjunctival-limbal graft. First degree (a) and third degree (b) of primary pterygium; (c) and (d) 1 day after surgery, the graft had been transplanted (green arrow). Subconjunctival hemorrhage was present around the graft; (e) severe and (f) moderate inflammation on conjunctiva 1 week after surgery, characterized by dilated vessels (yellow arrow); (g) and (h) 4 weeks after surgery, inflammation around the graft subsided and became mild, characterized by several dilated vessels (yellow arrow)

in the FD-AM group who previously had a moderate or severe inflammation at first week after surgery showed an improvement at the final follow-up. Postoperatively, six subjects with a previous moderate inflammation presented a mild inflammation, one subject with a previous severe inflammation presented had a mild inflammation, and three subjects with a previous severe inflammation presented a moderate inflammation. However, not all subjects showed improvement in the CLG group. At the final visit, three subjects with a previous moderate inflammation

showed no change in their condition, one subject with a previous moderate inflammation presented a mild inflammation, six subjects with a previous severe inflammation presented a mild inflammation, and two subjects with severe inflammation presented with a moderate inflammation.

Tear samples were collected before surgery (baseline) and at day-7 and day-28 after surgery. Table 2 presents the results. The IL-6 level on day-7 increased significantly in both groups compared with baseline ($p = 0.001$). The level of IL-6 on day-7 was

Table 2. Inflammation condition at day-7 and -28 after surgery in both groups

	Inflammation	Day-28					
		CLG (n)			FD-AM (n)		
		Mild	Moderate	Severe	Mild	Moderate	Severe
Day-7	Mild	1	0	0	3	0	0
	Moderate	1	3	0	6	0	0
	Severe	6	2	0	1	3	0

CLG=conjunctival-limbal graft; FD-AM=freeze-dried amniotic membrane

Table 3. IL-6 level in tears (pg/ml) and its change after surgery

	FD-AM, median (min-max)	CLG, median (min-max)	<i>p</i> *
Baseline	4.50 (1.82–11.79)	3.33 (1.32–12.91)	0.317
Day-7	27.22 (9.36–56.98)	36.42 (12.90–69.63)	0.980
Day-28	4.37 (2.19–26.43)	4.70 (1.64–15.42)	0.681
Δ IL-6 at day-7	22.85 (6.98–50.99)	23.51 (10.52–66.05)	0.778
Δ IL-6 at day-28	0.36 (–3.36–17.62)	0.32 (–4.48–10.42)	0.739

IL-6=interleukin-6; FD-AM=freeze-dried amniotic membrane; CLG=conjunctival-limbal graft

*Mann-Whitney test; Δ: change

relatively higher in the CLG group, although there was no difference compared to that in the FD-AM group.

DISCUSSION

The conjunctival inflammation after pterygium excision served as our primary measure. Currently, only a small number of studies have reported this outcome, and no study has explicitly compared the use of FD-AM and CLG in terms of conjunctival inflammation outcome. Kheirkhah et al,⁵ using cryopreserved AM on 24 eyes, revealed that the area under the graft had no inflammation at 3–4 weeks postoperatively; inflammation occurred in the surrounding conjunctiva in 62% of subjects. In this study, on day-28, although all the subjects still had a conjunctival inflammation, 23% of subjects in the AM group presented with a moderate inflammation, and none had a severe inflammation.

Kheirkhah et al¹² conducted a further research to compare a conjunctival inflammation between subjects who underwent AM and CLG procedure. A total of 37% and 26% of subjects in the AM group had a moderate and severe inflammation, respectively, at 1 month after surgery. Meanwhile, in the CLG group, 10% of subjects had moderate inflammation, and none had severe inflammation. The better results achieved in the CLG group might be caused by the different periods of suture removal between the two groups. In the AM group, the sutures were removed 2 weeks postoperatively, while

the procedure was performed 1 week postoperatively in the CLG group. Sutures can be considered as a foreign material in the conjunctiva and can elicit an inflammatory reaction. The duration for which the sutures were retained in the conjunctiva was positively correlated with the duration of the inflammation. In this study, no difference was observed in the period of suture removal between the AM and CLG groups; the sutures were removed 2 weeks postoperatively in both groups. This factor may explain why our study had different results than those reported by Kheirkhah et al.¹² Our study revealed that a moderate to severe inflammation was more common in the CLG group than the AM, although no statistical significance was there was no statistical significance.

Cha et al⁸ assessed a conjunctival inflammation after the pterygium excision and CLG transplantation and performed an intergroup comparison using fibrin glue or sutures. Seven days after surgery, all subjects (100%) with sutures presented with a moderate to severe inflammation, in contrast with the 43.3% subjects in the fibrin glue group. It is similar to that of the current study, which also used sutures and found 92% of subjects undergoing CLG had moderate to severe inflammation at day-7. Furthermore, Cha et al⁸ stated that on day-21 after surgery, the inflammation had reduced in both groups. However, the proportion was not specifically mentioned. In our study, both groups showed a reduction in the inflammation on day-28.

Suture was positively correlated with the incidence of inflammation after pterygium surgery compared with fibrin glue. Kheirkhah et al⁵ also observed that inflammation was more prevalent in subjects with sutures (61.5%) than in those with glue (21.4%) at 1 week after surgery. However, at week-4, both groups showed no difference in the severity of inflammation. This finding was probably caused by the removal of sutures at week-2 after surgery, in which suture as a factor responsible for eliciting inflammation has been removed. Other factors such as age, type of pterygium (primary versus recurrent), and duration of intraoperative mitomycin use were not statistically significant. In this study, sutures were removed at the same period in the FD-AM and CLG groups to allow for adequate healing process (graft was successfully integrated to surrounding tissue). This phenomenon was proven by the absence of complications, such as slipped or lost graft, during the follow-up period. However, sutures removed more than 2 weeks after surgery would induce prolonged inflammation.

Lee et al¹³ found different concentrations of tears IL-6 between normal subjects and subjects who had dry eye syndrome. The mean IL-6 concentration in normal subjects was 6.97 (6.73) pg/ml, which was lower than that of the subjects with dry eye (12.12 [13.54] pg/ml) or subjects with dry eye and Sjögren syndrome (19.22 [20.11] pg/ml). Similar findings were also found in a study by Yoon et al.¹⁴ The concentration of IL-6 in normal subjects stated in these studies is similar to the baseline IL-6 in our work, that is, 4.50 and 3.33 pg/ml in the FD-AM and CLG groups, respectively. Dry eye is a condition associated with inflammation on the ocular surface. Therefore, a high level of tear IL-6 is expected in dry eye subjects.

In this study, IL-6 level was equivalent in both groups, in which its median showed no difference with the mean IL-6 of normal subjects reported by Lee et al¹³ and Yoon et al.¹⁴ One week after surgery, the IL-6 level increased in both groups, in which the median level of IL-6 in the CLG group (36.42 pg/ml) was higher than that of the FD-AM group (27.22 pg/ml). However, the IL-6 increment exhibited no intergroup differences, with a higher value of 0.66 pg/ml observed in the CLG group. Moreover, the level decreased to nearly the baseline level after 1 month after surgery, indicating that the inflammation process had subsided.

The anti-inflammatory effect of AM becomes possible through several mechanisms. AM produces

a matrix metalloproteinase (MMP) inhibitor, whose function is to inhibit the activity of MMP secreted by neutrophils and macrophages. The stroma of AM also produces a leukocyte proteinase inhibitor, a lactoferrin, and an antagonist of the IL-1 receptor; all of these serve as anti-inflammatory factors. Leukocyte proteinase inhibitor inhibits the activity of proteinase secreted by leukocytes, whereas a lactoferrin has a role in suppressing IL-6 level. IL-1, along with IL-6 and IL-8, are kinds of proinflammatory cytokines.^{6,7,10}

The anti-inflammatory effect of AM is also supported by Lee et al,¹¹ whose study showed that the supernatant of AM has an effect on suppressing inflammatory mediators, such as IL-6 and IL-8, *in vitro*. In our study, we collected the patient's tears three times. Therefore, the IL-6 level was measured at three time points: pre-surgery 1 week, and 4 weeks after surgery. No measurement was conducted at the 24 hours as Lee et al¹¹ did. However, the increase in IL-6 level at day-7 indicated that the inflammation process continued until at least this time point. Four weeks after surgery, its level nearly reduced to the baseline value, suggesting that the inflammation process had subsided. However, most subjects in both groups continued to clinically present with a mild inflammation. If IL-6 was applied as one of the indicators of inflammation, its level was expected to be above normal at a certain time. This condition might be explained by the fact that many cytokines, and not only IL-6, are involved in inflammation, such as IL-1, IL-12, IL-18, tumor necrosis factor, MMP, and interferon gamma.^{6,7} Further research is necessary to determine the concentration of several proinflammatory cytokines, specifically those involved in or associated with pterygium surgery.

To the best of our knowledge, this research is the first study to compare FD-AM and CLG in terms of conjunctival inflammation after pterygium surgery. Previous studies used cryopreserved AM, which requires storage with a temperature of -80°C . Therefore, the usage of AM is limited in developed countries. We also measured the IL-6 level in tears as a parameter of inflammation. The limitation of this study is the difficulty in masking the conjunctival inflammation scoring based on clinical photographs. This dilemma is caused by relatively different manifestations between the two groups after surgery. Therefore, the assessor can perform assessments on whether one subject belongs to the FD-AM or CLG group.

In conclusion, the use of FD-AM after pterygium excision yields milder inflammation in comparison with CLG on day-7 and day-28 after surgery. Both groups showed similar increase in the IL-6 levels on day-7 and similarly returned close to baseline value on day-28, but no difference among them.

Conflict of Interest

Grace Wangge is editorial board member but was not involved in the review or decision process for the article.

Acknowledgment

We would like to acknowledge the Faculty of Medicine, Universitas Indonesia for supporting this research through Hibah PITTA UI 2018 program.

Funding Sources

This study was funded by Hibah PITTA UI 2018.

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