Original Article

Effect of Oral Riboflavin and Direct-sun UV-A on Tumor Necrosis Factor Alpha (TNF-•) Concentrations in Tears of Mild-Moderate Keratoconus Patients

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ABSTRACT

Purpose: To investigate Tumor necrosis factor alpha (TNF-α) levels in tears of mild-moderate keratoconus patients after high-dose oral riboflavin and direct sun UV-A exposure.

Study Design: Quasi-experimental study.

Place and Duration of Study: Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada, and Dr. Sardjito General Hospital, Yogyakarta, Indonesia, from December 2022 until April 2023.

Methods: The TNF-α from tear of 8 patients (10 eyes) was evaluated before and after therapy. All patients received a high dose of oral riboflavin (800 mg/day) and direct UV-A exposure (≥15 minutes/day) for a total of three months of follow-up. The TNF-α expression in tears, visual acuity and the risk factors were examined at baseline until three months follow-up.

Results: TNF-α levels in tears decreased significantly from baseline (6.74±1.39 pg/ml) to1st month (5.98±0.69 pg/ml; p=0.048). However, in the following months, the levels of TNF-α elevated in the 2nd month (6.29±0.84; p=0.390) and the 3rd month (6.20±0.95; p=0.177). Although re-elevated after two months, TNF-α concentration still decreased from the beginning of the study (6.74±1.39 to 6.20±0.95 pg/ml). The Uncorrected visual acuity and best corrected visual acuity were significantly improved from baseline to the last follow up (p = 0.02 and p = 0.026 respectively).

Conclusion: There was a significant decrease of TNF-α levels in tears of mild-moderate keratoconus patients 1st month after therapy but no significant decrease in the subsequent follow-up. Furthermore, UCVA and BCVA values were improved.

Key Words: TNF-α, Keratoconus, riboflavin, Corneal cross linkage.

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INTRODUCTION

Keratoconus is the most common cause of corneal transplantation in developing countries. It is characterized by thinning of stroma and conical changes in cornea. Keratoconus is a progressive disease that causes thinning of cornea, irregular astigmatism and severe visual impairment.[1] It is a multifactorial disease in which external environment

and genetic factors play an important role.[2] Previous studies have mentioned risk factors for keratoconus, such as habit of eye rubbing, history of atopy, exposure to sunlight, exposure to toxins (such as cosmetic use and industrial workers), age, race, family history and history of contact lens use.[3,4]

Global prevalence of keratoconus ranges from 1:500 to 1:2,000 of population. It appears in second or third decade of life, affecting quality of life and socio-economic status of the sufferers.[1]Morphology of the cone in cornea is characterized by thinning in the central part and thickening in the peripheral part, as well as changes in the expression of extracellular matrix components such as collagen, fibrin and laminin.[5] Biochemical changes, changes in cellular homeostasis, hormone-metabolic changes and changes in corneal inflammatory factors constitute pathophysiology of keratoconus.[6] Although keratoconus is known to be non-inflammatory, recent studies show that there are high levels of inflammatory factors, including TNF-α, Interleukin-1 alpha(Il-1α),Interleukin-1 beta (Il-1β), Interleukin-6 (IL-6) and Matrix metalloproteinase 9 (MMP-9), as well as decreased Lysyl Oxidase (LOX) and collagen IVA1, in tears. TNF-alpha levels are increased in tears, serum, corneal and epithelial tissue in keratoconus patients.[7] Studies also show that eye rubbing caused an increase in TNF-α levels.[8]

One of the major complications of keratoconus include impaired visual acuity.[9] In addition, structural and molecular changes in cornea cause corneal weakness, which increases the possibility of perforation.[9] Keratoconus management aims to improve the quality of life by focusing on improving vision, stopping progression, restoring shape and strengthening cornea.[10]

Corneal Collagen cross-linking (CXL), is an alternative therapeutic option that has been extensively researched and developed worldwide for the treatment of keratoconus.[11] CXL stabilizes cornea by strengthening corneal bonds and stopping progression of keratoconus.[12] CXL is performed with Riboflavin (vitamin B_2) and Ultraviolet-A (UV-A) light exposure to strengthen bonds between corneal tissues through a photosensitization process and changes in the structure of stromal collagen. Thus, making cornea more stable and resistant to enzymatic degradation.[12]Previous studies stated that CXL also reduced levels of pro-inflammatory factors, thereby reducing the rate of keratoconus progression. Levels of proinflammatory cytokines, including IL-1, IL-6, IL-17 and TNF-α, decreased in the tears and corneal tissue of keratoconus patients after CXL therapy.[7]

This research was based on the study conducted by John Jarstad by giving high doses of riboflavin orally and sun exposure as an alternative action for keratoconus therapy.[13] This study resulted in stability of cornea and a decrease in the degree of keratoconus after six months of treatment.[13]This research is still preliminary and opens up new insights for clinicians in treating keratoconus. Oral administration of riboflavin is promised as an alternative to CXL, where the chemical reaction between riboflavin and UV-A light can repair the structure of cornea and reduce the levels of inflammatory factors, i.e., TNF-α, which is high in keratoconus patients. It needs to be studied further to support the scientific evidence. This study aims to evaluate the TNF-α levels in mild-moderate keratoconus patients with high-dose oral riboflavin and direct sun UV-A exposure therapy.

METHODS

This was a non-randomized quasi-experimental study conducted in patients with mild-moderate keratoconus. The study was approved by the Medical and Health Research Ethics Committee Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada, and Dr. Sardjito General Hospital, Yogyakarta, Indonesia (ID: KE/FK/1563/EC/2022). A total of 9 patients (10 eyes) were examined. All patients received a high dose of oral riboflavin (800 mg/day) and direct UV-A exposure (≥15 minutes/day) for a total of 3 months of follow-up from December 2022 until April 2023. All the mild-moderate keratoconus patients who attended the eye center of Dr. Sardjito Hospital, Yogyakarta, who were willing to participate in the study, fulfilled the inclusion and exclusion criteria and agreed to take part in the study were recruited and signed an informed consent form. The inclusion criteria for this research were patients diagnosed with mild-moderate keratoconus by Amsler-Krumeich grading. This diagnosis was confirmed by the corneal subdivision at eye center, Dr. Sardjito Hospital, Yogyakarta. Other criteria were age ≤ 30 years, never been diagnosed with other eye diseases (such as corneal ulcer, glaucoma, retinal disease, etc.) and not currently receiving or never received CXL therapy before.

Data from all eligible patients were collected.

Uncorrected visual acuity (UCVA) and Best-corrected visual acuity (BCVA) were performed. Slit lamp bio-microscopy, Autorefraction, and keratometry were done to confirm the diagnosis. Meanwhile, the degree of keratoconus was assessed and graded based on the Amsler-Krumeich Keratoconus Grading. Before therapy, the patient was thoroughly examined for baseline values of tear TNF-α expression. Data such as age, gender and history of allergies were also recorded. Informed consent was taken. Tear samples were retrieved using Schirmer paper, with a minimum scale of 15 mm and the sample was stored in the Integrated Research Laboratory (LRT), Faculty of Medicine, Public Health, and Nurse, Universitas Gadjah Mada. The patients were administrated with 800 mg/day of riboflavin tablets for 30 days. The patients were instructed to sunbathe when the weather was sunny for at least 15 minutes/day between 09:00 and 15:00. Sunny weather was categorized into three; sunny without clouds, sunny with few clouds, sunny with lots of clouds but the sunlight was still translucent. Patients were also advised to record drug adherence and the type of sunny weather in the logbook on daily basis. After one month, the patients came for re-examination, which was repeated for up to three months.

Tear samples were collected using sterile methods without stimulation or anesthetic drops. Tears were sampled using Schirmer I method with filter paper. The paper strips were stored at −80°C until further use. The paper strips were thawed and eluted overnight at room temperature using 0.5M NaCl and 0.5% Tween 20 containing 0.05M phosphate-buffered solution (pH: 7.2). The volume of tears obtained was calculated by considering 1 mm of a wet Schirmer strip to contain one µl of tears. Thus, the end concentration of the eluted solution corresponded to a 20-fold dilution of the original tear sample. TNF-α levels were measured using a commercial TNF-α sandwich-type enzyme-linked immunosorbent assay (ELISA) kit (Eli Kine™ Human TNF-α ELISA Kit; Addkine; China).

Data were analyzed using SPSS (SPSS Inc., Chicago, Illinois, USA, Ver 21). Numerical variables were reported as mean ± standard deviation (SD) and median (mid-quartile range), while categorical variables were reported as frequency (percentage). $P < 0.05$ was considered statistically significant. The normality test of acquired data was done by Shapiro-Wilk test. Pre and post-therapy data were compared using repeated measurement paired t-tests for normally distributed data and Wilcoxon for non-normally distributed data. The Spearman correlation coefficient and t-test were used to compare the two groups regarding quantitative variables.

RESULTS

There were 62.5% females and 37.5% were males. Initially, there were 12 eyes of 9 people, but one person (two eyes) dropped out, so the total sample was ten eyes of eight people. The average age of the participants in this study was 17±8.17. Out of 10 eyes, eight were right (80%), and two were left (20%) eyes. All participants originated from Yogyakarta city, Indonesia (Table 1).

Table 1: *Baseline Characteristic.*

		Mean	Standard Deviation (SD)	N	%
Age (y-o)		17.13	8.17		
Gender	Male			3	37.5
	Female			5	62.5
Eye	Right			8	66.7
	Left			2	33.3
History of	Yes			4	50
Allergy	No			4	50

TNF-α levels in the tears of mild-moderate keratoconus patients in this study decreased significantly from month 0 (baseline) to one month ($p = 0.048$), from an average of 6.74±1.39 pg/ml at the start of the study to 5.98±0.69 pg/ml. The levels of TNF-α did not decrease significantly in the 2nd month ($p = 0.364$) and the 3rd month ($p = 0.177$). However, from month 0 to the end of the study, the average decrease was from 6.74±1.39 pg/ml to 6.20±0.95 pg/ml. (Table 2).

UCVA and BCVA results were converted to log sMAR. UCVA from baseline (mean 0.83 ± 0.25) to 3rd month (mean 0.64 ± 0.33) experienced a significant improvement ($p = 0.02$), and from 1st month (mean 0.80 ± 0.26) to 3rd month, there was also a significant increase in UCVA ($p = 0.06$). Continuing from 2nd month (mean 0.72 ± 0.33) to 3rd month, there was a significant increase ($p = 0.06$). BCVA also improved significantly, with significant improvement already seen from baseline (mean 0.20 ± 0.17) to the first month follow-up (mean 0.14 ± 0.12) with $p = 0.034$. BCVA also improved from baseline to 2nd month (mean 0.11 ± 0.12) with $p = 0.024$ and from

Table 2: *Changes of TNF-α levels, UCVA, and BCVA.*

Variable	month	0	1	2	3	*p* 0-1	0-2	0-3	1-2	1-3	2-3
†) TNF-α	Mean	6.74	5.98	6.29	6.20	**0.048***	0.364	0,177	0.390	0.322	0.839
	SD	1.39	0.69	0.84	0.95						
‡) UCVA	Mean	0.83	0.80	0.72	0.64	0.081	**0.019***	**0.002***	0.057	**0.006***	**0.037***
	SD	0.25	0.26	0.33	0.33						
‡) BCVA	Mean	0.20	0.14	0.11	0.10	**0.034***	**0.024***	**0.026***	0.083	**0.046***	0.317
	SD	0.17	0.12	0.12	0.09						

Note: *) significantly p<0,05, †)*Paired T-test*, ‡)*Wilcoxon*

Table 3: *The correlation between gender and TNF-α levels.*

TNF-α	Gender				P-value
	Male		Female		
	Mean	Standard Deviation	Mean	Standard Deviation	
Baseline	7.27	1.47	6.51	1.41	0.465
Month 1	6.47	.78	5.76	.58	0.143
Month 2	6.56	.93	6.17	.85	0.534
Month 3	6.77	.86	5.95	.93	0.229

baseline to 3rd month (mean 0.10 ± 0.09) follow-up with *p = 0.026*.

Independent T-test was used to see correlation between gender and history of eye allergies to TNF-α levels. The analysis found no significant correlation between TNF-α levels and gender (Table 3) or previous history of allergies (Table 4).

Table 4: *The correlation between allergy history and TNF-α levels.*

TNF-α	Gender				P-value
	Male		Female		
	Mean	Standard Deviation	Mean	Standard Deviation	
Baseline	6.45	1.72	7.03	1.10	0.544
Month 1	5.92	.62	6.04	.82	0.800
Month 2	6.08	1.01	6.50	.69	0.467
Month 3	5.74	1.03	6.66	.64	0.127

Pearson correlation was used to evaluate correlation between age and TNF-α levels each month. The analysis found no significant correlation between TNF-α levels and age (Table 5).

Table 5: *The correlation between age and TNF-α levels.*

	r	P-value
Month 1	0.619	0.056
Month 2	-0.122	0.737
Month 3	0.408	0.241

Spearman test was used to see correlation between the amount of sun exposure by looking at the total brightness of the day and analyzing the levels of TNF-α each month. The analysis results found no significant correlation between TNF-α levels and total sunny weather per day each month (Table 6).

Table 6: *Correlation of total sunny weather and TNF-α level.*

	r	P-value
Month 1	-0.029	0.936
Month 2	0.110	0.762
Month 3	0.283	0.428
Total sunny weather	-0.323	0.363

DISCUSSION

The mean TNF-α level at the beginning of the study (baseline) was 6.74 pg/mL. Although many studies have not mentioned normal levels of TNF-α in tears, but comparison between normal patients and keratoconus patients showed that TNF-α increased up to threefold in keratoconus patients.[14,15] This is consistent with the theory that cornea is an integrated part of ocular surface that contains specific and non-specific immune molecules. Tissue degradation, such as thinning in keratoconus, leads to expression of inflammatory mediators such as proinflammatory cytokines.[14]

In this study, TNF-α levels decreased significantly in the first month, reaching 5.98 pg/mL (p = 0.048). TNF-α levels decreased on average compared to baseline in the second (5.29 pg/mL) and third (6.20 pg/mL) months, although this was not statistically significant. In contrast to this study, Acar et al, reported a decrease in TNF-α expression (p = 0.002) in tears after three months of Collagen cross-linking (CXL).[16] Another study stated a significant difference in TNF-α levels in control patients compared to patients who received CXL therapy.[17] Another study on 80 samples consisting of a control group of 28 persons, keratoconus group of 32 patients and a CXL

group of 20 patients reported a significant decrease in TNF-α levels in patients receiving CXL therapy compared to those not receiving therapy.[7]

Recent research indicates involvement of several inflammatory factors, enzymes and cytokines such as Matrix metalloproteinase 9 (MMP-9), IL-6, cathepsins and TNF-α, which were increased in tear samples of Keratoconus patients.[18] TNF-α acts as a pro-inflammatory cytokine created due to inflammatory reactions, infections and environmental stresses. When a reaction occurs, TNF-α interacts with its receptors, Tumor necrosis factor receptor 1 (TNFR1) and Tumor necrosis factor receptor 2 (TNFR2), initiating the caspase cascade and activating two transcription factors, namely Activator protein-1 (AP1) and NF-kb. Cytokines therefore induce an immune response, which includes activation of TNF-α itself, IL1, IL-6, and IL-8, as well as adhesion molecules, including Vascular cell adhesion molecule 1 (VCAM-1) and Intracellular cell adhesion molecule 1 (ICAM-1), which leads to the accumulation of leukocytes at sites of inflammation.[19]

This event causes a degradation of the extracellular matrix, which results in changes in tissue architecture, induces cell migration and affects cell adhesion.[20] Increased TNF-α levels induce interleukins, especially IL-6 and Matrix metalloproteinase (MMP), which significantly affect inflammation, degradation, extracellular matrix and wound healing.[21] Although required for wound healing, unbalanced elevated levels of these substances over a long period can cause extensive degradation of the extracellular matrix and lead to corneal weakness and the progression of keratoconus.[20] In accordance with the hypothesis and theory, the decrease in TNF-α levels that occurs in the first month can be caused by processes similar to CXL, namely exposure to radiation intensity and UVA total energy absorbed by the cornea. Still, in the second and third months, there is no decrease in levels again, which needs to be studied. However, a longer follow-up time is needed to determine the next trend in TNF-α levels.

A correlation test of the frequency of sun exposure (sunny weather) on TNF-α levels also found no relationship between the number of sunny weathers per month and TNF-α levels. The correlation test between age and TNF-α levels also did not show a correlation where the sample age range was 7–33 years. It is in accordance with the theory that the incidence of keratoconus is in the 5 – 40 years range.[2] Literature shows that allergic conditions greatly affect TNF-α levels and the progression of keratoconus.[1] However, this study did not find a correlation between TNF-α levels and a history of allergies.

In this study, UCVA and BCVA values were improved. This is consistent with the previous study that evaluated six eyes of three keratoconus patients treated with oral riboflavin and UVA sunlight for six months.[13]

Limitations of this study were a single-center research with a small sample size, a short follow-up and lack of comparison. This research is still new and needs further investigation, with more samples, comparison and more follow-up. If this research can continue and develop into an alternative therapy, it will be cost-effective for healing keratoconus.

CONCLUSION

There was a statistically significant decrease in TNF-α levels in tears of mild-moderate keratoconus patients compared to baseline and 1st-month follow-up but not significant at second and third follow-up. However, the average of TNF-α levels decreased from baseline to the end of follow-up (three months). Furthermore, TNF-α levels had no correlation with age, gender, history of allergies and total sun exposure (sunny days). According to the CXL therapy for keratoconus research, this alternative therapy resulted in statistical improvements in UCVA and BCVA from baseline to the end of follow-up (three months).

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Conflict of Interest: Authors declared no conflict of interest.

Ethical Approval: The study was approved by the Institutional review board/Ethical review board (KE/FK/1563/EC/2022).

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