Advances in Biology & Earth Sciences
Vol.8, No.2, 2023, pp.187-195

IMPROVING IMMUNITY INDICATORS IN EYEWASH WITH AKTIPOL IN KERATOCONUS

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Abstract. ***Background*:** Up to date, the occurrence of degenerative changes in the cornea in keratoconus is a consequence of patient's impaired immune status. Since there are few data in this regard, we conducted a study in patients with keratoconus to establish the immune status of patients, and the changes in that after treatment with a drug with an immunomodulatory effect, Aktipol, as well. ***Methods*:** The study was conducted on 28 patients with keratoconus in the Central Research Laboratory of the Azerbaijan Medical University. All patients with keratoconus underwent parabulbar Aktiprol administration. Data obtained duting monotherapy were compared with the data of same patients before treatment and control (healthy of the same age). ***Results*:** An analysis of the cellular and humoral links of immunity revealed a shift in immune homeostasis association at all four stages of the disease. The changes we have identified at the systemic level of the immune status in patients are consistent with modern ideas about the immune concept of the pathogenesis of this pathology. ***Conclusions:*** A corrective impact of Aktipol on cellular and humoral immunity in keratoconus allows one to use it as a therapeutic agent for disorders of immune homeostasis in eyes as an immunomodulator.

Keywords: *Aktipol, eyewash, immunomodulator, keratokonus.*

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Received*: 20 May 2023;* ***Accepted****: 13 June 2023;* ***Published****: 8 August 2023.*

1. Introduction

The researchers state that immune inflammation is an important link in the pathogenesis and course of keratoconus (Asimellis & Kaufman, 2022; Espandar & Meyer, 2010; Gordon-Shaag *et al*., 2015), so finally, immune homeostasis disorders (Cagliari et al., 2022; Loh & Sherwin, 2022), allergic manifestations (D'Souza, 2021; Galvis *et al*., 2015; Tarasova *et al.*, 1996), inflammatory processes precede disease Fischer *et al.*, 2021; Ahmed *et al*., 2020). There are reports about the use of some immunomodulatory drugs for the keratitis treatment (Mazzotta *et al*., 2018). Nevertheless, the etiopathogenesis of keratoconus remains unknown (Sharma *et al*., 2013; McMonnies, 2015; Shetty *et al*., 2017; Maudgal *et al*., 1982). Most recently, an effective drug Aktipol (0.007% solution of para-aminobenzoic acid) has been introduced into the ophthalmology practice (Sharma *et al*., 2013). It has been

How to cite (APA):
Abdullayeva, A.M., Mamedova, V.M., Nasirova, V.B., & Amirova, M.F. (2023). Improving immunity indicators in eyewash with Aktipol in keratoconus. *Advances in Biology & Earth Sciences, 8*(2), 187-195.

established that aktipol is an inducer of secretion endogenous interferon, which applied locally, models the interferon status in patients with ophthalmic herpes (Akberova & Galbinur, 2000).

It is acknown that interferon inducers are a new generation amid immunomodulators that affect cellular and humoral immunity (Ershov &Tazulakhova, 1999).

Taking into account the immunopathology role in the keratoconus pathogenesis and the novel interferon inducer Aktipol properties, we set a goal to investigate this drug therapeutic effect in keratoconus conservative treatment.

The aim of this study was to study of Aktipol local application impact on the immune status of patients with keratoconus.

2. Material and methods

Study design

Immunological studies were carried out on 28 patients with keratoconus in the Central Research Laboratory of the Azerbaijan Medical University. The investigation was carried out on patients of Azerbaijani nationality aged 12 to 44 years, of which 2 aged 12 to 14 years (7.1%), 6 - 15 to 19 (21.4%) 12 - 20 to 29 (43%), 6 - 30 to 39 (21.4%) and 2 over 40 years (7.1%) patients. The control group consisted of 20 clinically healthy individuals aged corresponding to the age of patients with keratoconus. All patients with keratoconus underwent parabulbar Aktiprol administration at a dose of 0.5 ml, along with this; instillations of this drug were prescribed 2 drops 3-4 times a day during the course of treatment 14 days limited to 7 injections of Aktipol every other day. All patients during the study did not take any other drug, but Aktipol.

Immunoassay

During the **immunological study**, a set of methods was carried out to determine the cellular and humoral immunity indicators levels in the peripheral blood. We tested two important types of lymphocytes. Assessment of T-system cellular immunity state were carried out by reactions of spontaneous rosette formation (Ershov & Tazulakhova,1999; Novikov & Novikov, 2009).

When setting up the definition of B-lymphocytes, the methodological recommendation of A.N. Cheredeev A.N. was taken into account (Semenzato *et al*., 1978)

Humoral immunoassays include: 1) Y.Manchini reaction for the concentration of serum immunoglobulins (Akberova & Galbinur, 2000; Vorobyeva *et al.,* 2000) for the concentration of serum immunoglobulins; 2) quantitative determination of circulating immune complexes (CIC) carried out by the M. Digeon method modified Yu.A. Grinevich (Grinevich, 1981) serum complement activity determination by F.Yu. Garib and L.N. Sharapov method (Garib & Sharapov, 1973). Definition of the blood lysozyme activity was carried out by the nephelometric method according to V.G. Dorofeichuk (Dorofeychuk, 1968).

Statistical analysis

The results of the study were processed statistically. For comparison and probabilistic assessment of the control and the compared group indicator differences

(P), the Wilcoxon (Mann-Whitney) non-parametric paired rank U-test was used. For differences between pre-treatment and post-treatment indicators within groups (P), the paired Wilcoxon T-test (Lakin, 1990) was determined.

For each indicator 1st, 2nd and 3rd groups were distinguished. The first group included patients whose value before treatment was below the norm, the second group comprised patients with indicators within the normal range, and the third included patients with indicators exceeding the control before treatment.

3. Results and discussion

As a result of systemic immunity analysis of patients data with keratoconus, the violation of immune homeostasis was established; the changes relate to both cellular and humoral immunity.

The results on aktipol monotherapy treatment are visible via the examples presented in the figure below (Fig. 1).

Fig.1. Improving vision by Aktipol immunocorrection (elimination of injurious factors) with subsequent improvement of the vascular network

Clinical - immunological parallel will be carried out in our future work.

Table 1. Changes in cellular immunity indicators in patients with keratoconus (M±m (min-max))

Parameters	Below Norm		Within Norm		Above Norm		Control (N=20)
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	
T-lymphocytes, %	n_1=11 43,4±2,15 (26-50)	n_1=11** 54,9±1,66 (42-60)	n_2=16 59.4±130 (51-70)	n_2=16** 63,5 ± 0,78 (60-70)	n_3=1 87	n_3=1 72	63,8±1,85 (51-79)
T-helpers, %	n_1=2 16;20	n_1=2 24; 25	n_2=25 33,8±1,63 (21-25)	n_2=25** 39,52±1,18 (30-52)	n_3=1 62	n_3=1 54	40,1±2,03 (21-56)
T-suppressors, %	n_1=13 18,2±0,74 (10-20)	n_1=13 17,8± 0,74 (10-20)	n_2=15 22,5±0,64 (21-30)	n_2=15* 23,8± 1,03 (20-32)	-	-	23,7 ± 0,65 (21-33)
T-help/T-supr.	-	-	n_2=26 1,57±0,78 (0,83-2,36)	n_2=26 1,82 ±0,086 (1,14-2,87)	n_3=2 2,48; 2,88	n_3=2 3,05; 5,4	1,72±0,097 (0,7-2,43)

Note: * - P <0,05; ** - P <0,01

The results of determining the percent of T-lymphocytes and their subpopulations before and after Aktipol are presented in Tables 1 and 2. The examined patients had different basal immune activity. So, according to Table 1, in 11 out of 28 patients with keratoconus (39.3%) the percent of peripheral blood T-lymphocytes was significantly low vs. control ($P<0.001$). In the rest 16 (57.1%) patients, the percentage of T-lymphocytes did not differ from in the control group. Only in one case (3.6%) the T-lymphocyte levels was elevated.

In 2 (7.1%) patients, the T-helpers percentage was significantly lower vs. control; in 25 (89.3%) patients, the average levels of T-helpers, was significantly different from normal mean ($P<0.05$); and only in one case (3.6%), the percent of T-helpers was significantly exceeded.

Regarding the T-suppressor percent, in 13 (46.4%) out of 28 patients it was significantly lower ($P<0.001$) than in the control. In 15 (53.6%) patients, the percentage of T-suppressors did not differ significantly from the norm. We did not find any increased incidence of T-suppressors in patients with keratoconus.

In 26 (92.9%) patients, the immune-regulatory index, namely helper-suppressor potential did not differ significantly from the control; only in two out of 28 (7.1%), the helper-suppressor potential reached 2.48; 2.88, which exceeded the norm significantly (Table 1). The established changes showed that a decrease in the total number of T-lymphocytes (in 39.3% of patients) and quantitative deviations of T-lymphocytes subpopulations reflect a violation of lymphopoiesis in patients with keratoconus.

After treatment with Aktipol, patients with a low T-lymphocytes percentage showed a significant increase ($P<0.01$) in this indicator compared with the baseline: $54.9 \pm 1.66\%$ vs. $43.4 \pm 2.15\%$.

Regarding T-lymphocytes, although within the normal range, but a statistically significant increase in this indicator after treatment was observed: $63.5 \pm 0.78\%$ vs. $59.4 \pm 1.30\%$ ($P<0.01$)

In the single patient with an increased percentage of T-lymphocytes, after treatment there was a decrease in this indicator to the norm: 72%.vs. 87%

T-helpers in two patients with a low level of this subpopulation of lymphocytes increased after treatment and reached values corresponding to the lower border of the norm: 24; 25 vs.16; 20 baseline.

In 25 patients with a normal T-helper levels, after treatment this indicator levels significantly differed from the initial one. Although the changes in T-helper levels in the second group did not go beyond the normal range, however, the average value changed upwards with a 99% of confidence: $39.52 \pm 1.18\%$ vs. $33.8 \pm 1.63\%$. In the only patient with an increased T-helper levels, after treatment with Aktipol there was a decrease to the upper norm border: from 62% to 54% respectively.

According the patients with a reduced T-suppressor levels, after Aktipol administration, there was no significant difference from the initial level in this indicator: 18.2 ± 0.74 and 17.8 ± 0.74 respectively.

In patients with a normal percent of T-suppressors, its average level after treatment, even remained within the normal range, was statistically different (higher) from the initial level: 23.8 ± 1.03 vs 22.5 ± 0.64. ($P<0.05$) (Table 1).

The immunoregulatory index in the group of patients with its normal level before and after treatment remained within the normal range: 1.57 ± 0.078 and 1.82 ± 0.086, respectively.

In two cases with an increased helper-suppressor potential, after treatment, an increase of this index to maximum values was observed: from 2.48; 2.88 to 3.05;5.4.

An analysis of immunity humoral link parameters at keratoconus (Table 2) showed that the peripheral blood B-lymphocytes prcentage in 7 (25%) out of 28 patients was below the norm (P<0.001). The mean of this indicator in 21 (75%) patients, even within the normal range, but significantly (P<0.01) differs from the control group.

Against this background, in most patients, there was found a decrease in the blood serum concentration of immunoglobulin classes G, A, M (1gG, IgA, IgM). Thus, in 25 (89.3%) out of 28 patients, the immunoglobulin G levels was significantly lower than in the control group, with significance P<0.001 (Table 2). In three (10.7%) patients, the IgG levels was higher than norm (P<0.001).

In 10 (35.7%) out of 28 patients, the IgA levels was significantly reduced vs. control group (P<0.001).

In the remaining 18 (64.3%) patients, the IgA levels remained within the normal range, although there was not significant difference in mean value (P<0.01).

As for IgM, its level was reduced in 17 (60.7%) patients, with significance level. In 8 (28.6%) patients, this indicator remained unchanged, although the mean value was significantly different from the control with 99% confidence. The increase in IgM levels above the norm (P<0.001) was found only in three cases (10.7%).

It is noteworthy that the total percent of B-lymphocytes was reduced only in 25% of patients with keratoconus, while a decrease in various immunoglobulin classes was observed in 36-90% of patients.

A significant (P<0.001) decline in the CIC formed from antigens, antibodies and complement components was found in 9 (32.1%) patients. In six (21.4%) out of 28 patients, the CIC levels remained within the normal range. There was a significant increase in the CIC (P<0.001) compared with the control in 13 (46.5%) patients. This increase most probably indicates an antigenemia in keratoconus.

Changes were also recorded in the complement system responsible for the lysis and phagocytosis, that reflects a violation of homeostasis. In 15 patients with keratoconus (53.6%), the blood serum complement total hemolytic activity was significantly lower than in controls at 99% confidence level. In 13 (46.4%) patients of this group, the complement hemolytic activity did not differ from that in the control group (Table 2).

These changes in the complement system and lymphopoiesis can explain the allergic manifestation often accompanying keratoconus.

The next important immunological marker, a serum lysozyme activity index was above the norm in patients with keratoconus, or found at the maximal border of the norm. So, in 24 (85.7%) patients this index was within the normal range (P<0.001); while in four (14.3%), it was significantly higher than in the control group at 99% confidence. These changes often reflect the impairment of lysosomal processing.

After treatment with Aktipol, in the group with a reduced percentage of B-lymphocytes, a significant increase in this indicator compared with the initial level was observed: $6.94 \pm 0.11\%$ vs $5.71 \pm 0.04\%$ (P<0.05). In the second group, where the B-lymphocyte levels was within the normal range close to lowest border (P<0.001), after Aktipol administration, the percent of B-lymphocytes increases and already did not differ from the normal level mean. (P<0.01).

As it has been established, the interferon inducer Aktipol had a modulating effect on the immunoglobulins concentration. Thus, in 25 patients with low IgG initial levels,

after treatment, there was a significant increase in this class of immunoglobulins in peripheral blood: 928.5 ± 16.9mg % and 787.6 ± 21.7 mg % in examined and control groups, respectively (P<0, 01).

Table 2. Changes in the humoral immunity parameters in patients with keratoconus (M±m (min-max))

Parameters	Below Norm		Within Norm		Above Norm		Control (N=20)
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	
B-lymphocytes, %	n_1=7 5,71±0,04 (5,6-5,8)	n_1=7* 6,94±0,11 (6,4-7,2)	n_2=21 7,1±0,18 (6,2-9,4)	n_2=21** 8,41 ± 0,14 (7,4-10)	-	-	8,16±0,25 (6-10,2)
I_gG mg %	n_1=25 787,6±21,7 (500-860)	n_1=25** 928,5±16,9 (700-1060)	-	-	n_3=3 1788,7±107,8 (1646-2000)	n_3=3 1396,7±108,4 (1230-1600)	1036,5±2054 (870-1250)
I_gA mg %	n_1=10 89,7±7,41 (47-117)	n_1=10** 123,6± 8,77 (77-154)	n_2=18 160,4±5,63 (126-200)	n_2=18** 186,1± 4,66 (154-222)	-	-	195,8 ± 8,55 (126-258)
I_gM mg %	n_1=17 92,7±5,77 (37-120)	n_1=17** 132,1±5,89 (60-153)	n_2=8 130,0±0 (130-130)	n_2=8** 163,9 ±3,88 (153-184)	n_3=3 212,0±0 (212-212)	n_3=3 196,0±8,08 (186-212)	161,4±4,2 (130-203)
CİC (total) U/ml	n_1=9 34,6±2,65 (22-47)	n_1=9** 48,4±3,66 (35-62)	n_2=6 68,5±4,21 (52-75)	n_2=6 64,3±0,49 (63-66)	n_3=3 112,9±9,14 (85-189)	n_3=3** 97,3±7,51 (72-154)	63,8±1,69 (52-78)
Complement, %	n_1=15 55,5±0,95 (49-64)	n_1=15** 58,5± 1,07 (49-65)	n_2=13 75,9±1,77 (65-87)	n_2=13 75,6±2,4 (56-87)	-	-	80,3±1,86 (65-95)
Lysozyme, %	-	-	n_2=24 56,5±1,31 (40-64,5)	n_2=24 54,3±0,62 (48,5-65)	n_3=3 67,3±1,65 (65-72)	n_3=4 59,8±3,01 (52-65)	50,92±1,31 (40-64,5)

Note: * - P <0,05
** - P <0,01

We have already noted that an increase in the IgG levels was observed only in 3 cases. After these patients treatment, the IgG levels has significantly lowered compared to the initial level, and reached the highest border of control values, namely decreased from 1788.7 ± 107.8mg% to 1396.7 ± 108.4 mg%. The interferon secretion inductor Aktipol exhibited also a stimulating effect on the IgA concentration. After treatment, the average concentration of IgA in the group of patients with low Ig A class significantly increased vs its initial level, increasing from 89.7 ± 7.41 mg % to 123.6 ± 8.77 mg % (P<0.01).

In 18 patients with IgA concentration within the normal range but the arithmetic mean lower than that in the control, after treatment there was a significant increase in this class of Ig what results in achieving the mean close to control: indicator rised from160.4 ± 5.63 mg % to 186.1 ± 4.66 mg (P<0.01) .

In the group with low IgM levels equal 92.7 ± 5.77 mg % after treatment, there was found an increase in this indicator vs initial average value reaching 132.1 ± 5.89 mg % (P<0.01). In patients, whose IgM levels before treatment was on lowest border of the norm (130.0±0 mg %), after Aktipol administration, an increase of this

immunoglobulin class helped to reach the average value of norm (163.9±3.88 mg %) ($P<0.01$). In the group of patients with elevated compared to the control group levels of IgM, after treatment, there was a tendency to decrease in this indicator from 212.0 ± 0 mg % to 196.0 ± 8.08 mg %.

Aktipol treatment of patients with low total CIC concentration showed a significant increase in this indicator levels from 34.6 ± 2.65 U/ml to 48.4 ± 3.66 U/ml ($P<0.01$).

In the group with a normal CIC (total) concentration, after treatment with Aktipol, no changes in the immune complexes concentration were observed, i.e. neither increase nor decrease was noticed, the figures did not go beyond normal values.

In patients with elevated CİC levels, there was a significant decrease in this indicator vs the baseline, i.e. the immune complexes concentration drop from 112.9 ± 9.14 U/ml to 97.3 ± 7.51 U/ml ($P<0.01$).

A study of blood serum hemolytic activity of complement revealed the absence of its sufficient activity in half of the studied patients with keratoconus. Thus, the complement system of these patients showed insufficient activity, and this indicator remained low even after treatment with Aktipol: 55.5 ± 0.95% and 58.5 ± 1.07 % before and after treatment respectively ($P<0.01$).

As for patients with normal complement activity, their indicator also remained unchanged after treatment with Aktipol.

Serum lysozyme activity in patients with keratoconus was at or above the maximal normal border before treatment with Aktipol.

In the majority of patients (second group) after treatment, the normal serum lysozyme activity slightly decreased falling from 56.5 ± 1.31 % to 54.3 ± 0.62 % ($P<0.05$). In the remaining four patients with high serum lysozyme activity, this indicator decreased from baseline after treatment with aktipol from 67.3 ± 1.65% to 59.8 ± 3.01%.

Thus, we found the availability of changes in the immune system of patients with keratoconus. These changes relate to both cellular and humoral immunity, with a greater severity of the latter. Our data are consistent with modern ideas about the immune concept of the keratoconus pathogenesis.

We are pioneers in revealing that Aktipol, when applied locally, exhibits a systemic immunomodulatory effect and leads to the normalization of impaired cellular and humoral immunity. In the case of an initially elevated or reduced serum immune system parameters, after treatment with Aktipol, a decrease was observed in the case of elevated and / or an increase in the case of reduced levels, whereas at normal initial values, the indicators either did not change, or modulation leading to the achievement of normal mean values occurred.

In our previously published studies (Akberova & Galbinur, 2000), it was reported that locally applied aktipol exhibited approximately the same modulating effect on the interferon status of patients with various forms ophthalmic herpes.

4. Conclusion

1. With a cone-shaped deformation of the cornea, violation of immune homeostasis characterized by the immunity cellular and humoral component changes were revealed.

2. Aktipol exhibits an immunomodulatory effect on the immune status of patients with keratoconus, providing an equal corrective impact on cellular and humoral immunity. Obtained data coincide with with modern idea suggesting the interferon inducers as immunomodulators.

3. Since актipol, when applied locally, exhibits a systemic effect on various aspects of the defense system, it can be suggested as a treatment tool in immune homeostasis disorders as an immunomodulator.

The study has been evaluated by the Ethics and Use of Clinical Evidence Committee of Azerbaijan Medical University and deemed not to require ethics approval.

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