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Original Article

Neopterin Levels and Immune Response in Autoimmune Uveitis in an Experiment

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Purpose: To study and compare the immune response and neopterin levels in the blood in experimental autoimmune uveitis (EAU).

Methods: A model of EAU was created in 30 Chinchilla rabbits. Intravenous and intravitreal injections of normal horse serum were administered for this purpose. Clinical examinations and blood tests were conducted on days 3, 7, 10, 14, and 21. The blood investigation included the determination of neopterin (NP) level, white blood cell counts, lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+$.

Results: The peak in white blood cell count was observed on days 7 and 10 (6.4 ± 0.4 g/L and 6.0 ± 0.3 g/L, respectively), lymphocytes on day 3 (68.3% ± 2.4%, 3.0 ± 0.2 g/L), $CD3^+$ on day 7 (64.9% ± 3.1%, 2,032.5 ± 91.2 cells/μL), $CD4^+$ and $CD16^+$ on day 10 (54.6% ± 3.8%, 2,462.3 ± 60.7 cells/μL and 21.8% ± 1.8%, 691.2 ± 37.1 cells/μL, respectively). All these values did not return to the initial ones. There was a gradual decrease in the $CD8^+$ count from day 3 (12.5% ± 1.1%, 142.8 ± 9.1 cells/μL) with a subsequent gradual return towards normal levels by day 21. NP levels incresed on day 3 (5.2 ± 0.7 nmol/L), sustained on day 7 (5.2 ± 0.8 nmol/L), and started to decrease from day 10 (4.25 ± 1.7 nmol/L) to 2.3 ± 0.5 nmol/L on day 21. The highest correlation was observed between clinical manifestations and NP with a correlation coeffient of 0.799 (95% confidence interval, 0.719–0.858), which was significantly stronger ($p < 0.05$) than the correlations with other immune response markers.

Conclusions: During the modeling of EAU, there is an active immune response and a rapid reaction of NP on inflammation. NP is a significantly more sensitive marker of intraocular inflammation than the immune response. It can serve as a predictor of the onset and development of EAU.

Key Words: Animal, Blood, Immunity, Neopterin, Uveitis

Uveitis refers to inflammation of the uveal tract. Anterior uveitis involves the iris and ciliary body, intermediate uveitis affects the vitreous, and posterior uveitis involves the retina and choroid [1]. Prompt ophthalmologic evaluation is crucial, as delayed treatment may result in irreversible vision loss [2]. Major complications include macular edema, optic nerve edema, and cataract formation [3–6]. Uveitis encompasses a diverse group of inflammatory ocular diseases and is a major cause of both legal and economic blindness. Its socioeconomic impact is comparable to that of diabetic retinopathy [7]. Most affected individuals

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are young and of working age. In high-income countries, uveitis ranks as the fifth or sixth leading cause of blindness, accounting for approximately 35% of cases [8–10]. Uveitis is classified on infectious or noninfectious [11]: infectious causes are common (30%–60%) in the low-income countries [12]; noninfectious uveitis can occur in case of systemic autoimmune disease and ocular autoimmune diseases [13].

The eye maintains immune privilege (IP) by regulating innate and adaptive immune responses through mechanisms such as immunological ignorance, peripheral tolerance, and an intraocular immunosuppressive environment [14–16]. Ocular IP was first described by Peter Medawar, who observed prolonged survival of skin grafts in the anterior chamber compared to peripheral locations [17]. IP protects against intraocular inflammation to preserve visual function [18].

Ocular tissues such as the uvea, the cornea, the conjunctiva, and periocular fascia, contain rich networks of innate immune cells (bone marrow-derived resident macrophages and dendritic cells) which, together with the parenchymal cells, secrete a wide range of mediators which underpin IP [19].

IP serves as a homeostatic mechanism preserving tissue function in organs with specialized roles and limited regenerative capacity, such as the eye and brain [20]. However, strong immune responses can overcome IP, rendering these tissues more vulnerable to collateral damage compared to fully immunocompetent tissues [20].

During autoimmune inflammation, dysfunction of the blood-ocular barriers, intraocular immune modulators, and regulatory T-cell induction occurs [21,22]. IP offers limited protection against uveitis, as it primarily maintains tissue homeostasis rather than preventing strong immunological attacks. In severe uveitis, both infection and immune responses can cause irreversible structural damage [23]. While IP protects the eye from environmental microorganisms, it also makes it vulnerable to autoimmune attacks by lymphocytes primed elsewhere [24]. Barrier disruption permits investigation of immune responses in peripheral blood, with a priority on noninvasive methods to avoid procedures that may exacerbate inflammation.

T cell–mediated immunity is crucial in the pathogenesis of autoimmune uveitis [18,25–31]. Additionally, uveitis pathogenesis involves the release of specific proteins, some of which may serve as biomarkers [32]. Despite numerous inflammatory biomarkers being investigated, many exhibit instability in biological fluids [33–36]. Therefore, identifying stable proinflammatory biomarkers is essential for disease diagnosis and monitoring. Several authors suggest neopterin (NP) as a potential biomarker due to its stability and association with cellular immunity activation [37–39]. NP can be easily detected in blood, plasma, urine, tears, aqueous humor, vitreous humor, and various other biological fluids and tissues [40,41].

NP is an organic compound belonging to the pteridine class of heterocyclic molecules [37,42]. It is a biomarker produced by activated macrophages and dendritic cells in response to stimulation by interferon γ (IFN-γ), a cytokine released during immune responses [43–46]. NP serves as an indicator of cellular immune system activation and plays a role in various physiological and pathological processes [47,48].

Due to the complexity of uveitis diagnosis, which often requires multiple examinations, identifying accessible biomarkers in biological fluids could aid in the early detection of intraocular inflammation. Timely diagnosis is crucial, as uveitis can lead to rapid vision loss. In this context, laboratory diagnostics of immune responses and inflammation biomarkers are essential.

Despite existing studies on the immune response [48–52] and NP levels [37,53] in experimental autoimmune uveitis (EAU), this study is significant as it aims to compare immune response with NP levels in blood, addressing the gap in this area. This will help evaluate the role of neopterin as an inflammation biomarker for early diagnosis and prediction of EAU.

Materials and Methods

Ethics statement

This study was approved by the Commission on Bioethics of Danylo Halytsky Lviv National Medical University (protocol no. 13) on December 15, 2023. All animal experiments were performed in compliance with the Law of Ukraine No. 3447-IV On the Protection of Animals from Cruelty (February 21, 2006), the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 123), the Declaration of Helsinki guidelines for the use of experimental

animals (1964–2000), and the Council Directive 2010/63/EU on the protection of animals used for scientific purposes.

Study design

This study involved 30 Chinchilla rabbits aged 90 to 120 days and weighing between 2.5 and 3.0 kg. They were housed and maintained under conventional vivarium conditions.

Two weeks before the experiments, the rabbits were in quarantine. An already known model of autoimmune uveitis [54,55] was modified for the study. EAU was induced using the method described by Kuryltsiv et al. [56]. All rabbits were presensitized by intravenous injection 1.0 mL of normal horse serum (Serum Equine Normal for bacterial culture media, Biolik Pharma) daily for 5 days. Ten days after the last injection, 0.1 mL of normal sterile horse serum was intravitreally injected into both eyes of rabbits.

Clinical examination and blood analysis were regularly performed till day 36: before the intravenous injection of normal horse serum (on day 0), before the intravitreal injection of normal horse serum, on days 3, 7, 10, 14, and 21 after intravitreal injection of normal horse serum.

The control group's blood parameters were based on measurements taken on day 0 of the experiment from the same rabbits.

NP level was determined using the Rabbit Neopterin ELISA Kit (MyBioSource Inc). The determination of leucocytes or white blood cells (WBC), lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$ levels in rabbit blood was performed by the peroxidase-antiperoxidase immunohistochemical method, utilizing a set of monoclonal antibodies produced by Novus Biologicals. The results were evaluated photometrically using the Stat Fax 2100 microplate immunoassay analyzer (Awareness Technology Inc). The ocular examination was conducted on the same days of the experiment, and symptoms of uveitis were assessed according to the Standardization of Uveitis Nomenclature (SUN) criteria [57,58].

Statistical analysis

For the statistical evaluation of the results, the following criteria and methods were used: mean ± standard deviation, unpaired *t*-tests for normally distributed data and Mann-Whitney *U*-tests for non-normally distributed data. Differences were considered statistically significant at $p < 0.05$ (by Student *t*-test) [59]. The normality of quantitative variables was checked using the Shapiro-Wilk test. Due to the non-normal distribution of data, the results were presented as the median and interquartile range. Comparisons of quantitative variables among more than two groups were performed using the Kruskal-Wallis test, followed by post hoc analyses using Dunn test [60]. All statistical comparisons were two-tailed, with the significance level set at $p < 0.05$.

To evaluate the association between the severity of clinical manifestations of EAU, neopterin levels, and immune response parameters, a correlation analysis was performed. The Pearson correlation coefficient and its 95% confidence interval (CI) were calculated. Correlation coefficients for different parameters were compared using the method of CI calculation for differences [61,62]. To account for multiple hypothesis testing, the achieved significance levels were adjusted using the false discovery rate method [63]. The critical significance level was set at 0.05.

The analysis of the research results was also performed using the EZR ver. 1.61 (a graphical user interface for R ver. 4.2.2, R Foundation for Statistical Computing) [64].

Results

On day 0 a blood test was conducted on all animals to assess the immunological cells and NP levels (control group). The mean WBC count was 4.4 ± 0.1 g/L. Lymphocytes accounted for 28.9% ± 0.7% of WBCs, with an absolute count of 1.3 ± 0.03 g/L. The proportion of $CD3^+$ T cells was 57.4% ± 0.3%, corresponding to 729.8 ± 18.7 cells/μL. $CD4^+$ T cells constituted 43.5% ± 0.3% of lymphocytes (552.5 ± 12.8 cells/μL), while $CD8^+$ T cells accounted for 13.8% ± 0.4% (171.9 ± 5.9 cells/μL). The percentage of $CD16^+$ cells was 15.3% ± 0.4%, with an absolute count of 194.8 ± 5.6 cells/μL. The mean serum NP concentration was 1.7 ± 0.2 nmol/L.

After the intravenous injection of horse serum, the body temperature of the rabbits was measured daily, and it remained within the normal range in all rabbits throughout all days. Additionally, stool was normal. Upon clinical examination of the eye, no pathological changes were found.

Futhermore, before the intravitreal injection of normal horse serum the same a blood test was performed. The

mean WBC count was 4.65 ± 0.7 g/L, and lymphocytes accounted for 29.3% ± 1.6%, with an absolute count of 1.3 ± 0.06 g/L. $CD3^+$ T cells was 57.9% ± 1.6% (746 ± 16.3 cells/μL), 44.0% ± 1.4% (564.4 ± 13.8 cells/μL) for $CD4^+$, 13.8% ± 0.4% (165.1 ± 8.9 cells/μL) for $CD8^+$, and 14.9% ± 1.3% (187.9 ± 9.9 cells/μL) for $CD16^+$. NP was 1.8 ± 0.2 nmol/L.

We conducted a statistical comparison of the data indicators at this stage of the study with the indicators of the norm (day 0) and the indicators of the onset of the disease (day 3). All indicators prior to intravitreal serum injection were not statistically significant compared to the norm ($p > 0,05$) and were statistically significant compared to the ones on day 3 ($p < 0,05$).

Ocular examination from day 3 revealed varying degrees of uveitis, with mixed injection, corneal edema, precipitates, aqueous flare, miosis, posterior synechiae, and vitreous cellular reaction. The average total score of clinical manifestations of intraocular inflammation was 9.1 ± 0.5. On days 7 and 10, the inflammation score was 10.3 ± 0.4 and 11.3 ± 0.1, respectively. Starting from day 14, a slow and slight regression of EAU was observed in some parameters. The inflammation score remained relatively high at 10.2 ± 0.2. However, from day 21, uveitis showed significant regression. This regression was characterized by a decrease in the number of cells in the anterior chamber and vitreous body, along with reduced corneal and iris edema (inflammation score, 4.9 ± 0.05).

An active immune response to inflammation was observed starting from day 3 of the experiment. The WBC count was 4.9 ± 0.3, 6.4 ± 0.4, 6.0 ± 0.3, 5.3 ± 0.7, and 5.3 ± 0.6 g/L on days 3, 7, 10, 14, and 21, respectively. These value ranges showed a statistically significant difference compared to day 0 ($p < 0.05$), indicating an increase in this parameter throughout the experiment. The peak in WBC count was observed on days 7 and 10. The lymphocytes count, as well as $CD3^+$, $CD4^+$, and $CD16^+$ markers, also increased starting from day 3. A peak in lymphocytes was observed on day 3 (68.3% ± 2.4%, 3.0 ± 0.2 g/L), while the overall count of $CD3^+$ peaked on day 7 (64.9% ± 3.1%, 2,032.5 ± 91.2 cells/μL), $CD4^+$ and $CD16^+$ markers peaked on day 10 (54.6% ± 3.8%, 2,462.3 ± 60.7 cells/μL and 21.8% ± 1.8%, 691.2 ± 37.1 cells/μL, respectively), followed by a gradual decrease (Fig. 1A–1E). However, they did not return to initial values.

Furthermore, there was a gradual decrease in the $CD8^+$ from day 3 (12.5% ± 1.1%, 142.8 ± 9.1 cells/μL) to day 10 (9.8% ± 0.8%, 138.0 ± 3.7 cells/μL), with a subsequent gradual return to normal levels by day 21 (Fig. 1). Consequently, the immunoregulatory index, representing the $CD4^+/CD8^+$ ratio, demonstrated an upward trend: 3.2 ± 0.1 on day 0 (baseline), 4.0 ± 0.3 on day 3, 5.4 ± 0.7 on day 7, 5.7 ± 0.9 on day 10, 4.9 ± 0.8 on day 14, and 3.7 ± 0.6 on day 21.

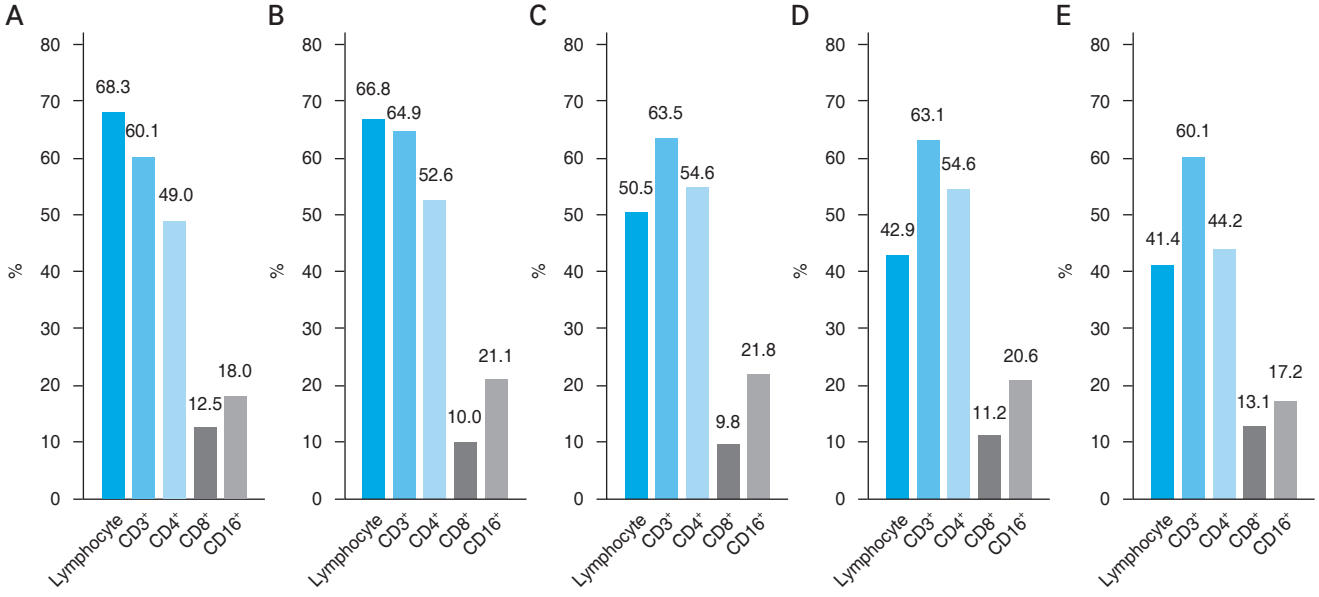

Fig. 1. Dynamics of percentages of immune cells in the blood serum of experimental rabbits with experimental autoimmune uveitis. (A) Day 3. (B) Day 7. (C) Day 10. (D) Day 14. (E) Day 21.

Regarding the NP level, a threefold increase was observed on day 3 of the study, reaching 5.2 ± 0.7 nmol/L. This level remained stable on day 7 (5.2 ± 0.8 nmol/L) and gradually declined from day 10 (4.25 ± 1.7 nmol/L) to 2.3 ± 0.5 nmol/L by day 21 of the experiment (Fig. 2) [65].

To assess the rate of change in all investigated parameters over time, the difference (Δ) in the level of each marker from its baseline mean level before the experiment (expressed as a percentage) was calculated using the following formula:

$$\Delta Xi\ (\%) = \frac{Xi - XBaseline}{XBaseline} \times 100$$

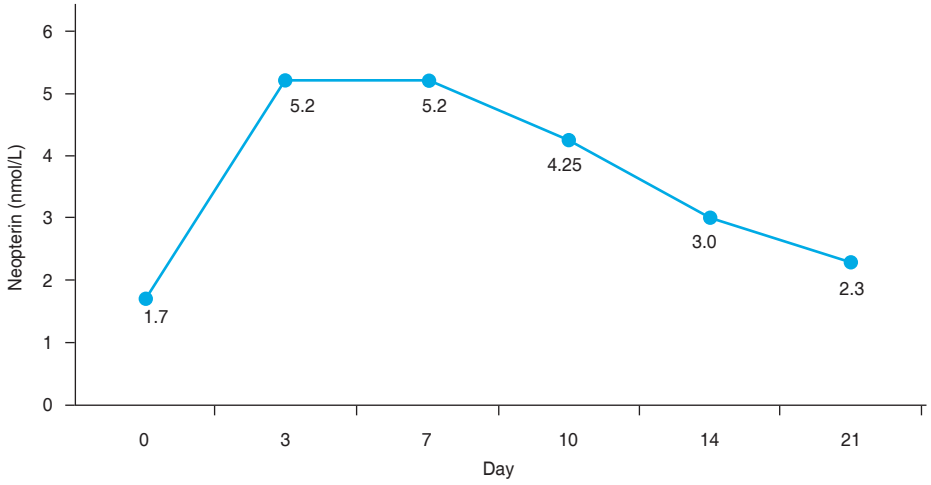

Fig. 2. Dynamics of the neopterin level in the blood serum of experimental rabbits with experimental autoimmune uveitis.

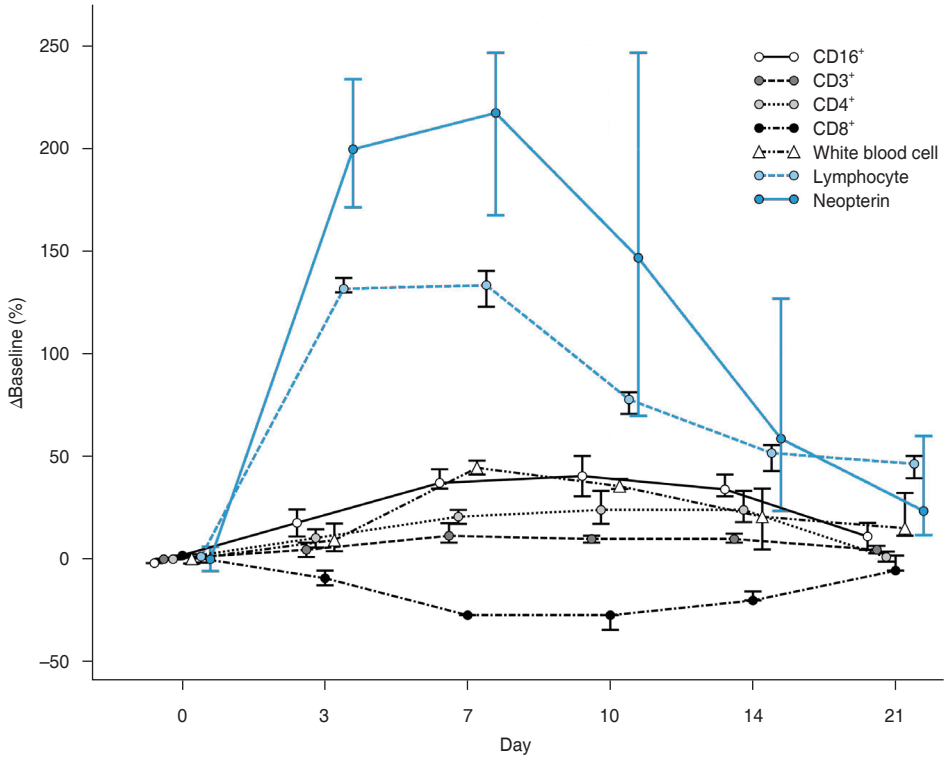

Fig. 3. Rate of change (Δ) of immune cells and neopterin levels in the blood serum of experimental rabbits with experimental autoimmune uveitis.

Table 1. Correlation coefficient of clinical manifestations with NP and the correlation coefficient of clinical manifestations with the immune response markers

Indicator	r (95% CI)	*p*-value*	Adjusted *p*-value†
NP	0.799 (0.719 to 0.858)	-	-
White blood cell	0.467 (0.305 to 0.602)	<0.001	<0.001
Lymphocyte	0.458 (0.296 to 0.595)	<0.001	<0.001
$CD3^+$	0.640 (0.514 to 0.739)	0.007	0.021
$CD4^+$	0.657 (0.535 to 0.752)	0.013	0.032
$CD8^+$	–0.411 (–0.556 to 0.241)	<0.001	<0.001
$CD16^+$	0.610 (0.480 to 0.718)	0.002	0.008

NP = neopterin; CI = confidence interval.
*Significance level of the difference between the correlation coefficient of clinical manifestations with NP and the correlation coefficient of clinical manifestations with the immune response markers; †Significance level of the difference between the correlation coefficient of clinical manifestations with NP and the correlation coefficient of clinical manifestations with the with the immune response markers, adjusted for multiple comparisons using the false discovery rate method.

As shown in Fig. 3, the NP level demonstrated the most rapid and pronounced increase. Other markers also responded to the development of intraocular inflammation during the experiment, albeit to a lesser extent and with a slightly delayed onset. Therefore, it was considered appropriate to further analyze the dynamics of each marker in relation to the dynamics of NP. The dynamics of lymphocytes and NP significantly differed from those of WBC, $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+$ cells in terms of their pattern ($p < 0.05$). Both WBC and NP reached their peak values on day 3 and began to decrease from days 7 to 10. On the other hand, the WBC, $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+$ markers peaked on days 7 to 10, with a decrease beginning on day 14. Thus, the levels of lymphocytes and NP significantly preceded changes in WBC, $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+$ cells by 4 to 7 days ($p < 0.05$).

A correlation analysis was performed for all measured parameters, and the correlation coefficient between immune response markers, neopterin levels, and the severity of clinical manifestations was determined. Table 1 presents correlation coefficients along with their 95% CIs. A comparison of the strength of the correlation between the severity of clinical manifestations and the parameters was performed.

To account for multiple comparisons and reduce the probability of type I error, the achieved significance level for differences between correlation coefficients was adjusted using the false discovery rate procedure [63]. For all pairwise comparisons, the adjusted p-value was <0.05, indicating a significant difference between the correlation coefficient r and other correlation coefficients (Table 1). Thus, the highest correlation was observed between clinical manifestations and NP with a correlation coeffient of 0.799 (95% confidence interval, 0.719–0.858), which was significantly stronger ($p < 0.05$) than the correlations with other immune response markers.

Discussion

We conducted a clinical and immunological study in rabbits following five intravenous serum injections to investigate the potential development of uveitis as a manifestation of serum sickness. Although cases of serum sickness-induced uveitis are rare, occurring in less than 0.5% of cases, it has been described in historical literature [66–68]. Anterior uveitis in serum sickness is triggered by the introduction of foreign serum or certain medications (e.g., antipneumococcus serum, penicillin, infliximab, azithromycin, streptokinase) [67–71]. Circulating immune complexes peak 7 to 10 days after foreign substance introduction.

In this experiment, no cases of uveitis developed as a manifestation of serum sickness based on clinical and blood analysis results. However, EAU developed in all rabbits following intravitreal serum injection, with active uveitis observed by day 3. An active immune response, consistent with earlier descriptions of T-cell activation, was evident during EAU development [72–75]. This response was characterized by elevated WBC, lymphocytes, and

$CD3^+$ cell counts, indicating the activation of self-reactive T cells. $CD3^+$ cells, which are normally regulated by immunogenic tolerance, play a central role in autoimmune diseases, including uveitis [76,77]. The balance between autoimmunity-inducing $CD4^+$ T cells and suppressor $CD4^+$ T cells is essential for maintaining immune regulation. Failure of this regulation leads to uncontrolled expansion of self-reactive T cells, resulting in autoimmune disease development [72]. Although in some previous publications, $CD8^+$ T cells increase during the course of experimental uveitis [78,79], the mechanism of intraocular inflammation in the experimental study is based on a regulation disruption. Evidence for this is the reduction in the number of $CD8^+$, i.e., cytotoxic suppressor T lymphocytes, which normally regulate the activity of $CD4^+$, i.e., helper T lymphocytes . Consequently, there is an increase in T helper activity. According to their findings, there is a reduction in the number of $CD8^+$ lymphocytes, but at the same time, the amounts of IFN-γ, interleukin 17 (IL-17), and IL-6 increase [80]. This suggests that the disruption of immune regulation contributed to the autoimmune response observed in EAU.

We also studied the NP levels in blood serum during EAU, as serum NP is a reliable marker of monocyte/macrophage activation [81]. Some studies report a negative correlation between NP levels and immune cell counts [82,83], while others associate elevated NP levels with active disease stages or autoimmune exacerbation [84,85]. In this study, NP levels rose significantly by day 3, increasing more than threefold in response to intraocular inflammation. Notably, the increase in NP and lymphocytes occurred faster than other immune cells (WBC, $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+$), and NP levels normalized more rapidly. This suggests that NP is a highly sensitive marker and may serve as a predictor for the onset of acute intraocular inflammation or recurrence of chronic uveitis [37].

As a result of the experiment, similar patterns of changes in lymphocytes count and NP levels were observed. However, as shown in Fig. 3, lymphocytes did not return to the normal pattern by the end of the experiment. This is confirmed by the statistically significant difference in the number of lymphocytes before the start of the experiment compared to their number at the end of the study (28.9% ± 0.7% on day 0 vs. 41.5% ±2.0% on day 21, $p < 0.05$). Furthermore, no statistically significant changes were observed in the neopterin level indicators (1.7 ± 0.2 nmol/L on day 0 vs. 2.3 ± 0.5 nmol/L on day 21, $p > 0.05$). Nevertheless, previous studies suggest that measuring NP levels in blood offers advantages over assessing lymphocytes count. While specific lymphocytes subsets, such as $CD4^+$ T cells, may increase in uveitis, total lymphocytes count does not reliably distinguish between active and inactive disease stages [86]. This is because systemic T-cell activation can occur without significant changes in peripheral blood lymphocytes levels. Additionally, specific immune activation markers, such as IL-2 receptor expression on T cells, are considered more indicative of active inflammation than total lymphocyte counts [86].

Lymphocytes, while important for understanding immune responses, may not specifically indicate uveitis without additional context, such as clinical signs or diagnostic criteria. Some studies highlight the inaccuracy of blood lymphocytes analysis and recommend using additional tools, like imaging techniques (e.g., optical coherence tomography) or inflammatory markers (e.g., neutrophil to lymphocyte ratio), alongside clinical assessment for accurate uveitis diagnosis and monitoring [87–89]. In this context, NP is a more sensitive and specific biomarker, as its levels consistently rise during active inflammation, directly reflecting immune system activation. Elevated NP levels indicate macrophage activation, which plays a key role in uveitis-related inflammation [90]. Additionally, a study showed that, despite no significant differences in serum interferon levels between patients and controls, NP levels were higher in patients with active disease, suggesting localized activation of the interferon system [91].

Although this study did not investigate NP levels in various biological fluids, the ease of measuring this biomarker in serum, plasma, urine, aqueous humor, tear fluid, and saliva underscores its significance in uveitis diagnosis [41,92, 93]. NP's high stability across different environments makes it a reliable marker for immune activation and inflammation [94]. This is crucial for monitoring immune responses in various clinical contexts. Additionally, our previous studies suggest varying NP levels in ocular environments based on the severity of anterior uveitis [41], though further investigation is needed.

According to the previous study, there is no data comparing the sensitivity of NP and the immune response to inflammation in the eye. In our study, we established a correlation between NP levels, immune markers, and clinical changes. This statistical analysis revealed a statistically

significant correlation between NP levels, immune response markers, and clinical signs of EAU. These findings allow us to conclude that NP is a significantly more sensitive marker of intraocular inflammation, responding rapidly to its development and precedes the onset of the immune response to the antigen in the autoimmune process.

In summary, NP is a highly sensitive marker of intraocular inflammation, responding rapidly to inflammatory changes in the eye. Its cost-effectiveness makes it a useful marker for noninfectious, postoperative autoimmune uveitis in the early stages, aiding in the prediction of exacerbations, guiding timely treatment, and monitoring disease progression and treatment outcomes.

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