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Detection of neopterin in tear samples

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Abstract: The main goal of the present study was to detect neopterin concentrations in human tear samples and to evaluate its potential correlation with serum neopterin levels. For this purpose, 20 systemically healthy volunteers were recruited, and both tear and serum samples were synchronically collected from each individual. Enzyme-linked immunoassay (ELISA) was carried out to detect the quantity of neopterin in the samples. Mean human tear neopterin levels were observed as 3±0.56 nM while mean serum neopterin was 9±1.25 nM. Additionally, a significant positive correlation between tear neopterin and serum neopterin concentrations was observed. This is the first report to show neopterin concentration in human tears as a biological sample. Collecting tears from the individuals is a non-invasive sampling method, and as an analytical aspect detection of neopterin by ELISA in tear samples construct a valuable, practical and cheap procedure for the diagnosis and monitoring of intraocular inflammation and systemic immune-mediated diseases.

Keywords: ELISA; neopterin; tear.

Introduction

Human cornea mostly consists of collagen and water which is enveloped by epithelium and endothelium layers. The layers collaborate to protect tissue homeostasis by providing adequate corneal transparency and reliability [1, 2]. The functions of moisturizing the ocular surface and minimizing damage to the corneal epithelium are maintained by tears. Human tears consists of electrolytes, water, mucin, lipids, and proteins including antibodies, lysozymes, lacritin, lipocalin, lactoferrin. These ingredients come together to form the outermost lipid layer, a middle aqueous layer, and the epithelium-covering mucoid layer [1, 3, 4]. Dysfunction in any of these layers can yield tear film instability and hyperosmolarity. External causes of such dysfunction are widespread including environmental factors, systemic diseases, and some medications [3, 5]. Tear production is currently evaluated by the Schirmer tear test, fluorescein clearance, and fluorescein tear break-up time [5]. A tear's pH is 7.4 the same as for blood, and the osmolarities of tears and serum are similar (298 and 296 mOsm/L, respectively) [1, 6]. It is thought that determination of biomarkers such as neopterin in tears might be useful for diagnosis and prognosis of disorders, especially immunopathological event-mediated ocular diseases.

Neopterin, a non-conjugated pteridine derivative with low molecular weight, is a sensitive biomarker of cellular immune response, and it is released from human monocyte/macrophages by the stimulation of interferon-gamma (IFN-γ). It is synthesized from guanosine triphosphate with the activation of T helper cells during immune response of type 1 [7–9]. Increase in neopterin concentrations are reported in several pathologies or physiological alterations, especially with enhanced monocyte/macrophage activity [10–12]. It is known that detection of neopterin levels in biological samples provides information about the cellular immune condition and help to predict the outcome and prognosis of various clinical conditions. Neopterin is biologically stable and can be easily quantified in human body fluids (for instance in blood and urine), and its concentrations in serum and in urine increase in parallel to the clinical course of infections with viruses, intracellular bacteria, infections [11–16]. A number of studies evaluated the neopterin status in various human biological samples, but not in tears.

The present study was mainly undertaken to determine human tears and serum neopterin concentrations in healthy volunteers. In this way, it was also aimed to evaluate whether neopterin tear levels, as a biomarker reflect the serum neopterin status or not.

Materials and methods

Subjects

Twenty healthy volunteers from the clinic staff were recruited for the study, and a short questionnaire was filled in by each subject to yield

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information on sex, age, and general health status. All of them had normal ophthalmological examinations, without any ocular inflammation. The study participants included 11 females and nine males with a mean age of 39 years. This study was performed with the Local Ethics Committee approval (#E-15-434/Feb. 19th, 2015), and Helsinki Declaration was followed during the study.

Sampling

Samples were taken in early morning for possible circadian variations. Blood samples were drawn into vacutainer tubes and centrifuged for 10 min at 1500 *g* at 4°C. Simultaneously, tear fluid was directly collected without anesthesia and any stimuli using eppendorf tubes. Sampling was done in the ophthalmology clinic.

Both biological fluids were kept away from direct light, and stored at –20°C until assayed. According to the kit prescription the adequate volume of tear or serum samples was directly pipetted in the plate after thawing. It means that no pre-treatment was applied to the stored samples.

Measurement of neopterin

Neopterin was determined by a commercially available neopterin enzyme-linked immunoassay (ELISA) kit (IBL, Hamburg, Germany) according to the manufacturer's instructions. The neopterin test principle based on the basic principle of a competitive ELISA and an unknown amount of antigen in the sample and a fixed amount of enzyme labeled antigen compete for the antibody-binding sites (rabbit-anti-neopterin). Both antigen-antibody complexes bind to the wells of the microtiter strips coated with a goat-anti-rabbit antibody. Unbound antigen is removed and the intensity of the color developed after the substrate incubation is inversely proportional to the amount of antigen in the sample. The optical density was measured by using ELISA microplate reader (Sunrise, GmbH, Tecan, Austria). All samples were measured in duplicates. The obtained optic density of the standards were plotted against their concentrations, the formula 4 parameter logistics transformation was used in order to obtain linearization (R^2=0.998), and then unknown neopterin content of the samples were calculated. The neopterin results were expressed as nanomolar (nM).

Statistical analysis

The results were expressed as mean standard error of the mean (SEM). The correlation between the tears and serum neopterin results was performed by Spearman's rank correlation analysis with p-value <0.05 indicating statistical significance.

Results

The neopterin concentrations of the subjects were presented in Table 1. The mean tear neopterin concentration was 3.0±0.56 nM ($\bar{x}$=2.18; min=0.73, max=9.91). Mean value of serum neopterin concentrations was 9.0±1.25 nM ($\bar{x}$=7.30; min=4.04, max=24.14).

Table 1: Tear and serum neopterin concentrations of the healthy individuals.

Sample	Volunteer	Age, years	Gender	Neopterin, nM	
				Tear	Serum
NG15-1	NY	60	F	2.22	15.22
NG15-2	YB	74	F	9.91	20.35
NG15-3	CD	41	M	1.24	10.19
NG15-4	HY	27	F	2.14	6.08
NG15-5	EG	32	F	4.30	17.32
NG15-6	MG	41	M	n.d.	4.16
NG15-7	HY	40	F	1.48	4.04
NG15-8	BG	40	F	1.32	4.90
NG15-9	AB	40	M	7.79	7.42
NG15-10	GC	38	F	2.74	5.54
NG15-11	NP	38	F	2.74	11.90
NG15-12	BE	30	M	n.d.	7.18
NG15-13	OC	37	M	0.73	6.88
NG15-14	MO	29	F	1.88	5.31
NG15-15	CK	23	M	1.53	7.51
NG15-16	NG	54	F	2.41	7.89
NG15-17	SU	42	F	2.57	8.81
NG15-18	KA	47	M	0.97	7.05
NG15-19	AK	24	M	2.45	24.14
NG15-20	AC	27	M	1.32	6.95

n.d., Not detected because of the insufficient sample volume (<20 μL). F, Female; M, male.

As shown in Figure 1, tear neopterin concentrations were positively correlated with serum neopterin levels (R_s=0.500, p=0.04).

Discussion

Tear components have always been used in diagnosis, and prognosis of some ocular diseases and the therapeutic

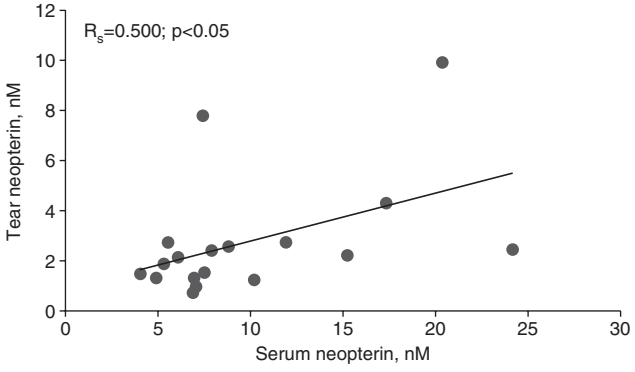

Figure 1: The correlation between tear neopterin and serum neopterin concentrations.

schemes [6]. In ophthalmologic pathologies, such as uveitis, Sjögren's syndrome, serum and/or urinary and/or saliva neopterin levels were evaluated [13–15]. Although tears are not similar to the other biological fluids, xenobiotics such as halogenated chemicals, methanol and some drugs can be excreted into tears and detected in this fluid [1, 3, 4, 6]. It is also known that baseline tears are derived not only from main and accessory glands but also from plasma infiltrate of conjunctival vessels.

In the literature there are few studies to show neopterin levels in lens material and aqueous humor samples [17]. Unfortunately, these sampling methods are done during operation and that is why it can be regarded as an invasive procedure. When the eye is stimulated, reflex secretion from the main lacrimal gland is activated. In this study, the tears were collected from lid margin tear meniscus, via capillary tubes without touching the conjunctiva. That is why the sampling mainly reflects basal secretion values here. The present study aimed to test the likely utility of tear neopterin as a biomarker of cellular immune activation for use in wild and free-ranging settings in ocular disorders. Sampling was done with conventional methods without any pre-treatment procedure, which is another advantage for this study.

The eye holds a privileged immunological status, in contrast to most compartments in the body. Its immunology is known to consist of numerous local and systemic effects [18]. Tears form an important element of local immunity of the eye, as having one of the first and earliest elements of natural immunity. Its composition also results in powerful antibacterial, antiviral and antifungal effects [19]. Recently, Santacruz et al. reported how microbial pathogens can modulate local and systemic immune responses and clinical appearance and demonstrated distinctive cytokine profiles in the tears of patients with microbial keratitis. The study has shown a predominance of IL-8 (interleukin-8) over IL-6 in tears of patients with bacterial keratitis, Gram-positive and -negative. In addition to that, patients with Gram-negative keratitis have a higher frequency of circulating (CD3−CD56+) natural killer cells and IL-1β in tears. Their study subjects with fungal keratitis express IL-8, IL-6, and IL-1β in tears without changes in circulating cells [20]. The clinical and toxicological importance of pteridines, notably neopterin, that have roles in various biochemical pathways have been reported in various studies [10–17]. The evaluation of neopterin is used in progress of the diseases as well as in diagnosis and treatment. It is known that to use biomarkers primarily the basal levels of healthy individuals are needed for future comparisons. Until today, there is no data and studies on tear neopterin concentrations, except for a paper in Russian by Onishchenko et al. in which higher tear neopterin compared to serum neopterin was reported in their control group; however, no correlation between serum and tear neopterin levels was observed [21]. In the present study, data collection for the mentioned tear neopterin levels has been initiated. Our results for tear neopterin are promising, as neopterin is present in detectable concentrations in tear samples. Additionally, tear neopterin levels were significantly correlated with those in serum, even with three-fold differences. In addition to that, it can be expected to have enhanced tear neopterin levels, as well as other biologic fluids, in exposure to infection agents, inflammatory factors and chemical determinants.

In conclusion, this is the first study in English to show neopterin levels in tear samples and it might be concluded that, consideration of tear neopterin together with traditional ocular local tests will be clinically useful in early diagnosis of ocular pathologies. Detection of neopterin levels by ELISA technique with low sample volume in tear fluid seems to be a useful, simple, cheap and non-invasive diagnostic tool. According to the data the neopterin concentrations should be confirmed by studies which consist of patients with ocular inflammatory disease.

Conflict of interest statement: All authors have declared no conflicts of interest. All authors contributed to the manuscript and approved its final version.

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