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Determination of advanced glycation end-products and antibodies against anti-CML and anti-CEL in the serum of Graves' orbitopathy patients before and after methylprednisolone treatment

Advanced glycation end-products and antibodies against anti-CML and anti-CEL in the serum of Graves' orbitopathy

Ocena stężenia produków zaawansowanej glikacji białek i przeciwciał anty-CEL i anty-CML w surowicy pacjentów chorujących na orbitopatię Gravesa przed i po leczeniu metyloprednizolonem

Produkty zaawansowanej glikacji białek i przeciwciała anty-CEL i anty-CML w surowicy pacjentów z orbitopatią Gravesa

Janusz Strzelczyk[1], Magdalena Szumska[2], Aleksandra Damasiewicz-Bodzek[2], Anna Krywult[4], Michał Długaszek[3], Justyna Czubilińska[4], Kaja Gawlik[4], Konrad Synowiec[3], Krystyna Tyrpień-Golder[2], Karolina Poczkaj[1], Beata Kos-Kudła[1]

[1]Division of Endocrinology, Department of Pathophysiology and Endocrinology, Silesian Medical University, Katowice, Poland
[2]Department of Chemistry, Faculty of Medicine and Division of Dentistry, Medical University of Silesia, Zabrze, Poland
[3]Member of Student Research Society, Department of Chemistry, Faculty of Medicine and Division of Dentistry, Medical University of Silesia, Zabrze, Poland
[4]Member of Student Research Society, Division of Endocrinology, Department of Pathophysiology and Endocrinology, Medical University of Silesia, Katowice, Poland

Abstract

Introduction: The glycation process is a non-enzymatic modification of proteins occurring due to the reactions of reductive carbohydrates. The glycated residues lose their biological functions, and their removal process is ineffective. They accumulate, and as a result they cause an immunological response. The aim of this study was a determination of the concentrations of advanced glycation end-products and antibodies against carboxymethyl lysine (anti-CML) and carboxyethyl lysine (anti-CEL) in the sera of Graves' orbitopathy patients.
Material and methods: The study group were patients from the Division of Endocrinology of the Medical University of Silesia (n = 25) suffering from Graves' orbitopathy. The concentration of AGE-peptides using flow spectrofluorimetry method, and anti-CML and anti-CEL IgG antibodies using immunoenzymatic technique (ELISA), were measured in patients sera before and after methylprednisolone treatment.
Results: In sera of the study group the concentrations of AGE-peptides and anti-CML were significantly lower before and after treatment in comparison to the control group ($p < 0.05$). Mean values of anti-CEL concentrations were comparable (at both phases of treatment) with the value observed in the control group. After treatment the concentrations of AGE-peptides and anti-CEL significantly decreased ($p < 0.05$); however, the concentration of anti-CML was also lower but the observed change was not significant ($p > 0.05$).
Conclusions: In the course of Graves' orbitopathy the glycation process is disturbed. The treatment modifies significantly the process by lowering the concentration of advanced glycation end-products and suppressing the immune response to them.
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Key words: *Graves' orbitopathy; methylprednisolone treatment; anti-CML; anti-CEL*

Streszczenie

Wstęp: Glikacja jest nieenzymatyczną modyfikacją białek zachodzącą z udziałem cukrów redukujących. Produkty glikacji białek tracą swoje biologiczne funkcje, przez co ich usuwanie staje się nieefektywne. Kumulują się one, a jako neoepitopy wywołują odpowiedź immunologiczną. Celem pracy była ocena stężeń końcowych produktów zaawansowanej glikacji białek (AGE-peptydów) i przeciwciał przeciwko karboksymetylolizynie (anty-CML) i karboksyetylolizynie (anty-CEL) w surowicy chorych na orbitopatię Gravesa.
Materiał i metody: Badaną grupę stanowili pacjenci Kliniki Endokrynologii Śląskiego Uniwersytetu Medycznego (n = 25) cierpiący na orbitopatię Gravesa. W surowicy krwi żylnej oznaczono zawartość AGE-peptydów przy użyciu metody spektrofluorymetrii przepływowej oraz przeciwciał anty-CML i anty-CEL w klasie IgG przy użyciu techniki immunoenzymatycznej (ELISA), przed i po leczeniu preparatem metyloprednizolonu.

Janusz Strzelczyk M.D., Division of Endocrinology, Department of Pathophysiology and Endocrinology, Silesian Medical University, Katowice, Ceglana 35, Poland, phone/fax: 32 35 81 366, e-mail: januszstr@op.pl

Wyniki: W próbkach surowicy grupy badanej wykazano istotne statystycznie niższe stężenia AGE-peptydów i anty-CML zarówno przed, jak i po leczeniu w porównaniu z grupą kontrolną ($p < 0,05$). Średnie stężenia anty-CEL były porównywalne (w obu etapach leczenia) do obserwowanych w grupie kontrolnej. Po zastosowanym leczeniu stężenie AGE-peptydów oraz anty-CEL uległo znaczącemu obniżeniu ($p < 0,05$); stężenie anty-CML również było niższe, lecz zaobserwowana różnica nie była znamienna statystycznie ($p > 0,05$).
Wnioski: W przebiegu orbitiopatii Gravesa procesy glikacji białek ulegają zaburzeniu. Na podstawie wyników wstępnych badań można stwierdzić, że leczenie istotnie modyfikuje ten proces, zmniejszając stężenie końcowych produktów zaawansowanej glikacji oraz hamując odpowiedź immunologiczną przeciwko nim. **(Endokrynol Pol 2016; 67 (4): 383–389)**

Słowa kluczowe: *orbitopatia Gravesa; terapia metyloprednizolonem; anty-CEL, anty-CML*

Introduction

Graves's orbitopathy (GO) is the most severe complication of Graves-Basedow disease (GD), which has a significant impact on the quality of the patient's life [1]. In more than half of the patients symptoms subside spontaneously, but about 3–5% require aggressive treatment due to the severe course of GO [2]. The extrathyroidal symptoms of GD, like GO, are the most difficult issues in the course of this pathological state. Unfortunately, the available treatment is not always effective [3, 4].

Graves's orbitopathy is a chronic, autoimmune inflammation of orbital tissues, including eye muscles and periocular connective tissue. The role of immune system activation in its pathogenesis is doubtless and has been well documented [5–7, 8]. Activated T lymphocytes release cytokines and stimulate orbital fibroblasts to proliferate and produce glycosaminoglycans [9]. Recently, scientists have focused on the role of oxidative stress in the development of autoimmune disorders of the thyroid gland. Wilson et al. [10] showed increased free radicals and reduced antioxidant activity in active GD, and they also proved that an anti-thyroid medication modifies oxidative stress. Akarsu et al. [11] proved that the concentrations of malondialdehyde (MDA) — a marker of lipoxidation — were significantly higher in GO groups than in patients without ophthalmopathy and healthy controls, and the levels of glutathione - an important antioxidative agent - were significantly lower in the GO groups. Thyroid hormones also influence the synthesis and degradation of proteins [12]. The modification of protein structure may contribute to numerous pathological processes and can play a direct role in tissue damage.

Many researchers report an important role of a non-enzymatic modification of proteins caused by glucose, known as glycation. During that process, advanced glycation end products (AGEs) are formed by a non-enzymatic binding of free reducing sugars and reactive carbonyls to proteins. This reaction is called browning reaction or Maillard reaction [13]. The chemical structure of AGEs differs depending on the kind of amino compounds and sugars engaged in the process. N-ε–(carboxymethyl) lysine (CML) is the major known AGE produced by diverse reaction conditions [13, 14]. Other AGEs such as N-ε-(carboxyethyl) lysine (CEL), lactate lysine, pyrraline, pentosidine, and imidazoles are also frequently found, but also there are many others with unidentified structure and origin. Neoepitopes formed in glycation processes are recognised as foreign proteins by the B- and T-cell immune response [15, 16]. The process of AGE formation is intensified by hyperglycaemia or an oxidative stress [17]. The presence of AGEs is associated with a large number of various dysfunctions, including diabetes, atherosclerosis, renal failure, and neurodegenerative diseases [13, 17]. It is noteworthy that GD patients are more likely to suffer from diabetes and diabetic complications [18]. In autoimmune diseases, the role of AGEs and antibodies against them (anti-CML, anti-CEL) has also been investigated. In psoriasis, patients in an active phase of the disease have significantly higher concentrations of AGE-peptides, as well as anti-CML and anti-CEL antibodies, than healthy individuals [14]. In contrast, serum AGE concentrations in chronic spontaneous urticarial (CSU) patients were significantly lower as compared with the healthy subjects [19]. Plasma soluble receptor for advanced glycation end products (sRAGE) may serve as a potential biomarker for disease activity and a future therapeutic target in systemic lupus erythematosus (SLE) [20].

There is no available data on the role of advanced protein glycation in the pathogenesis of GO. Studies on animals have shown that the levels of the glycation product N-ε-fructose lysine (FL) and CML identified by GC/MS in liver proteins decreased significantly in hyperthyroid rats, proving that thyroid hormones influence this process [21]. Due to the increasing interest in the usage of various receptors for advanced glycation end products (RAGE) (especially those being expressed on the surface of monocytes) as potential therapeutic targets in the states of inflammation and autoimmunisation [20], like GO, it is essential to examine the process of glycation in that state.

The aim of the study was an estimation of the concentration of the peptides containing glycated

residues (AGE-peptides) and IgG antibodies against carboxymethyl lysine (anti-CML) and carboxyethyl lysine (anti-CEL) in the sera of patients suffering from GO before and after the treatment, in comparison to the sera of healthy control.

Material and methods

The study included archival sera samples from 25 patients suffering from Graves-Basedow orbitopathy, obtained in the SUM Endocrinology Clinic in Katowice. A study group consisted of 22 women and 3 men, (48.8 ± 8.8 years old), with mean BMI index 24.8 ± 4.4 kg/m^2. Patients were described by clinical activity index of orbitopathy (CAS, Clinical Activity Score), and most patients (n = 9) were classified as CAS = 6, and in NO SPECS classification most patients (n = 16) were in class 4. None of the patients suffered from diabetes. The control group consisted of healthy men (n = 19) and women (n = 21) aged 38.6 ± 9.0 years. Blood samples were taken in fasting from elbow veins of the study group twice: before and three weeks after applying the pulses of methylprednisolone. The treatment scheme was: six infusions of 0.5 g of methylprednisolone every two days. Sera obtained by centrifugation were stored at –80°C until the tests were performed. The study protocol was accepted by the Local Bioethical Commission of Silesian Medical University in Katowice. All participants were informed and signed the participation agreement.

The concentrations of IgG antibodies against carboxymethyllysine (anti-CML) and carboxyethyl lysine (anti-CEL) were determined using ELISA technique. ELISA plates (Maxisorp, Nunc, Denmark) were coated with the antigen solution at 10 mg/L in carbonate buffer. Antigens consisted of glyoxal derivative of human serum albumin (HSA-CML) and sodium pyruvate derivative of human serum albumin obtained in reducing conditions (HAS-CEL) [16]. The tested sera were diluted 800x in PBSTG (phosphate buffered saline with 0.1% Tween 20 and 0.1% gelatine) for IgGs titration and incubated in a plate at 37°C for 1.5 hours. IgG antibody class was detected using goat anti-human IgG conjugated with horseradish peroxidise (Sigma, USA). Incubation with conjugates was performed at 37°C for 1.5 hours. The reactions were developed using substrates containing O-phenylenediamine and H_2O_2 (for anti-CEL IgG) and tetramethylbenzidine (for anti-CML IgG). Absorbances were measured using a PowerWave XS Reader (BioTek, USA), and the results were calculated using KCJunior software (BioTek, USA). Calibrations were performed using pooled sera originating from approximately 100 healthy blood donors. 100-times dilution was accepted as 800 arbitral units/mL (AU/mL) for IgG antibodies (calibrating curve consisted of six standards: 25–800 AU/mL). This method has been previously described in the literature [14].

Measurement of advanced glycation end products (AGE-peptides) in tested sera was performed using flow spectrofluorimetry according to the method described by Zilin et al. [22]. Conditions were adapted for high-pressure liquid chromatography HPLC Ultimate 3000 equipment (Dionex, USA) with fluorescent detector RF 2000 (Dionex, USA). The obtained results were presented using basic parameters of descriptive statistics, such as mean value and standard deviation. The normal distribution of data was measured using Shapiro-Wilk's test. Wilcoxon's pair test was used to compare dependent data before and after the treatment. Independent data from the study group and the control group were compared using nonparametric Kołmogorow-Smirnow and U Mann-Whitney tests. $p < 0.05$ was considered statistically significant. Calculations were performed with STATISTICA 10.0 software (StatSoft, Cracow, Poland).

Results

The results of the study are presented in Table I. Patients suffering from GO showed significantly lower concentrations of AGE-peptides and antibodies against CML in comparison with the control group ($p < 0.05$). The differences in levels of antibodies against CEL were not significant ($p > 0.05$). After the treatment, concentrations of AGE-peptides and antibodies against CEL decreased significantly ($p < 0.05$), but anti-CML antibody levels remained unchanged ($p > 0.05$). These changes are shown in Figure 1, 2, and 3. It is noteworthy that mean anti-CEL antibody concentrations after

Table I. ***The concentrations of AGE-peptides, anti-CML IgG and anti-CEL IgG in patients with GO (before and after treatment) and in healthy controls***

Tabela I. ***Stężenia AGE-peptydów, anty-CML IgG, anty-CEL IgG u pacjentów z orbitopatią Gravesa (przed i po leczeniu) oraz u zdrowej grupy kontrolnej***

	Patients with GO		Healthy controls
	Before treatment	After treatment	
AGE peptides [mg/L]	17.4 ± 8.6*#	12.5 ± 6.2*	28.5 ± 15.4
	(5.6–40.5)	(4.8–25.7)	(12.7–67.8)
anti-CML IgG [AU/mL]	18.0 ± 30.1*	10.9 ± 10.1*	41.1 ± 31.3
	(3.2–148.7)	(2.1–47.1)	(8.6–135.9)
anti-CEL IgG [AU/mL]	70.9 ± 71.2#	53.2 ± 46.7	36.6 ± 34.5
	(8.1–242.3)	(7.7–198.4)	(8.7–145.1)

*$p < 0.05$ in GO patients *vs.* healthy control; #$p < 0.05$ in GO patients before treatment *vs.* after treatment

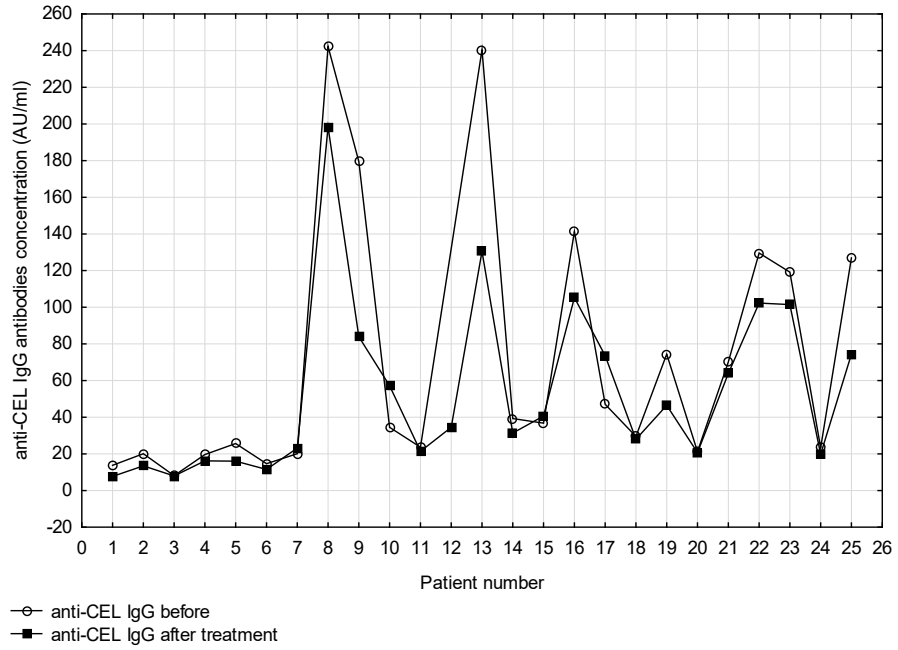

Figure 1. *Concentrations of anti-CEL IgG antibodies in serum of individual patients*
Rycina 1. *Stężenie przeciwciał anty-CEL IgG w surowicy pacjentów*

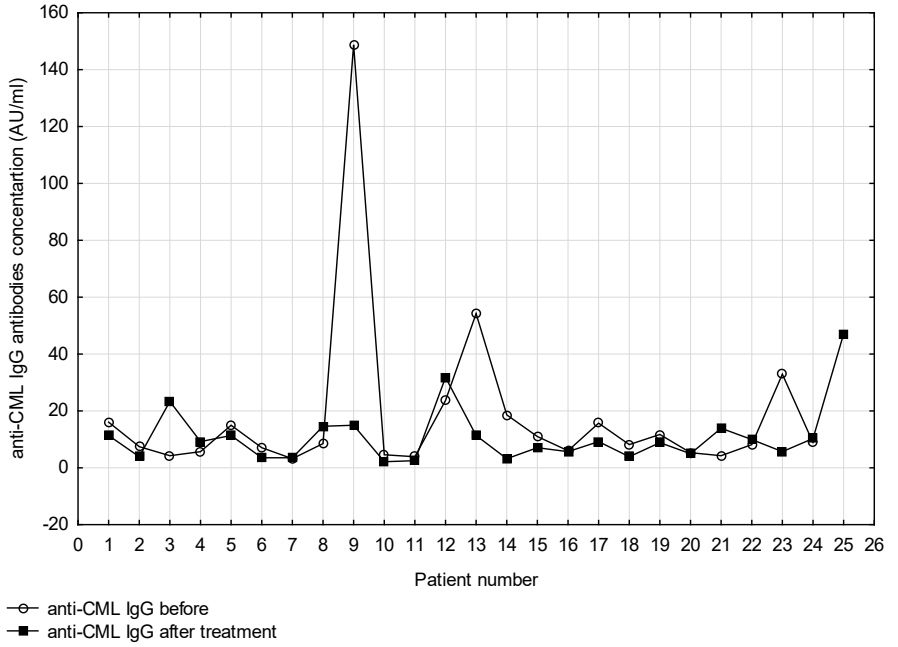

Figure 2. *Concentrations of anti-CML IgG antibodies in serum of individual patients*
Rycina 2. *Stężenia przeciwciał anty-CML IgG w surowicy pacjentów*

the treatment still matched those of the control group ($p > 0.05$), and anti-CML antibody and AGE-peptide levels were still lower ($p < 0.05$). The study did not reveal any differences in AGE-peptide, anti-CEL, and anti-CML values before and after the treatment with regard to NO SPECS or CAS classification. Table II shows correlations between the obtained values. AGE peptides concentrations show no significant correlation with anti-CEL and anti-CML neither before nor after the treatment ($p > 0.05$). AGE-peptides and anti-CEL values after the treatment decreased evenly due to significantly strong (AGE before & AGE after) and very strong (anti-CEL before & anti-CEL after) correlations. Antibodies against specific fragments of AGE-peptides revealed a moderate correlation ($p < 0.05$), but only before the treatment. Furthermore, the concentrations

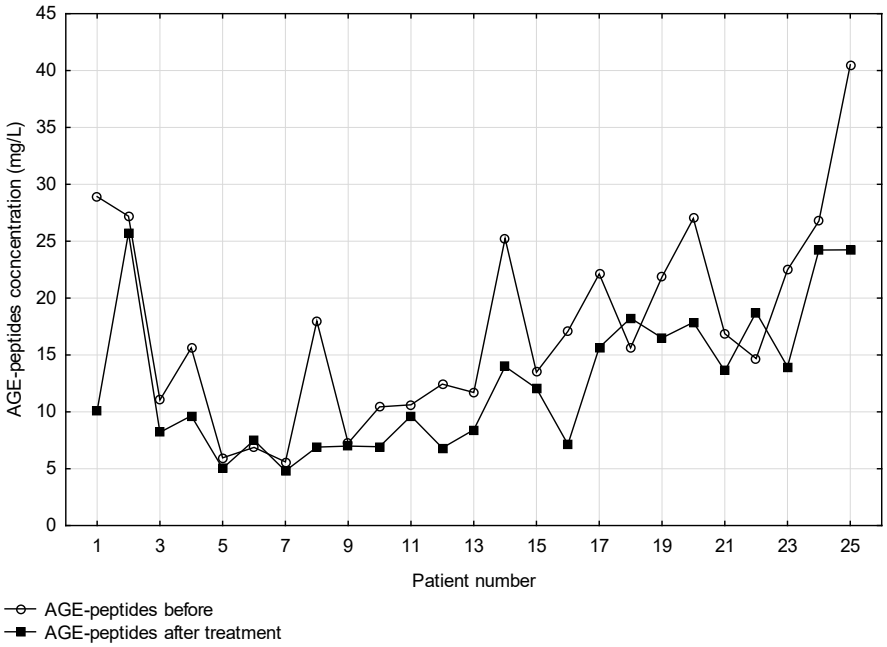

Figure 3. *Concentrations of AGE-peptides in serum of individual patients*
Rycina 3. *Stężenia AGE-peptydów w surowicy pacjentów*

Table II. ***The correlations between studied values***
Tabela II. ***Korelacje pomiędzy badanymi wartościami***

		R Spearman
AGE before &	AGE after	0.745*
	Anti-CEL before	0.069
	Anti-CML before	0.222
AGE after &	Anti CEL after	–0.036
	Anti-CML after	–0.076
Anti-CEL before &	Anti-CEL after	0.958*
	Anti-CML before	0.464*
Anti-CML before	Anti-CML after	0.388
Anti-CEL after	Anti-CML after	0.201

* $p < 0.05$ for statistically significant correlation

of AGE-peptides positively correlated with the age of the patients, both before (R = 0.517) and after (R = 0.481) the treatment.

Discussion

The process of protein glycation in the course of GO is certainly disturbed. AGE peptide concentrations in the sera of GO patients are decreased in comparison to healthy individuals. It can be explained that in autoimmune disorders, the elimination of AGE peptides occurs through endocytosis by macrophage's scavenger receptors, but it still requires further cytological studies [23]. What is more, the anti-CML concentrations were also decreased in GO patients in comparison to healthy subjects. In many autoimmune diseases increased levels of cytokines, like IFN-γ or TNF, might suppress the production and secretion of antibodies, which may impair the response to the AGE peptides [7]. Moreover, it is observed that the concentration of AGE peptides in serum is increasing with patient age. They might be cumulating in an organism throughout the ageing process because AGE peptides are long lasting molecules [24].

The study showed no significant correlation of the measured parameters before and after the treatment with the results of the NO SPECS and CAS classifications. These scales determine the severity of eye changes in Graves' disease based on the results of ophthalmic examination [25, 26]. The lack of correlation between obtained results and severity of GO could be explained by the natural history of GO. The primary demonstration of Th1 cells and connected cytokines (IFN-γ, TNFα, IL-1α) in the early stage of the disease indicates that cell-mediated immunity predominates in the initial phase of the disease [27]. In the subsequent phases of GO, immune response is predominantly demonstrated by Th2 cells and cytokines (IL-5, IL-4, IL-10, and IL-13), which stimulate B-cells to produce autoantibodies against TSH-receptor [27]. No links between severity of GO and studied proteins can be explained in another way. The treatment with glucocorticoids affects the regression of ocular lesions, although

the effects of such treatment are visible only after about 12 weeks [28]. Control examinations of studied patients were performed three weeks after the treatment, which could be an insufficient period of time to assess reliably the effectiveness of the glucocorticoid therapy.

In this study, the concentrations of AGE and anti-CEL levels have been significantly decreased in GO patients after the methylprednisolone bolus. In contrast to our data, in another study the methylprednisolone boluses increased glycaemia, so it should intensify the formation of AGEs in our patients [29]. This shows that the applied immunosuppressive treatment can affect the formation of glycated proteins or removal of studied proteins from the circulation.

Another study showed that immunosuppressive treatment has an impact on oxidative stress marker levels, so AGE distribution could be disturbed in GO patients after steroid boluses [30]. A non-significant decrease in the concentrations of anti-CML may indicate the existence of additional processes, which regulate the production of these antibodies. The existence of another pathway that regulates the production of anti-CML can be confirmed by the fact that there was a significant correlation between the concentrations of anti-CEL and anti-CML in patients before the treatment with glucocorticoids. After the immunosuppressive therapy this correlation was no longer observed**.** Moreover, the lack of significant correlation between the concentration of AGE peptides and linked antibodies (anti-CEL, anti-CML) shows that the immune response to AGE peptides in GO patients is not proportional to the concentrations of glycation products.

The pathogenesis of exophthalmos occurring in patients with Graves-Basedow is caused by three processes: cell infiltration, oedema, and fibrosis. Decreased levels of AGEs and analysed antibodies indicate the body's response to the immunosuppressive therapy. It would be necessary to study the role of receptors for AGEs in the course of GO, and investigate the possibility of using them as a therapeutic target in the treatment.

Conclusions

The study findings allow us to conclude that in the course of GO the glycation process of proteins is decreased or the products of the process are degraded and removed from the circulation. The use of an immunosuppressant modulates progression of the glycation and the intensity of the B-cell immune response against newly-formed neoepitopes. There have been no studies carried out before to investigate that phenomenon, and this study can improve the knowledge about pathogenesis of GO. Clinicians should be aware that treatment of GO affects not only the disease itself but also other processes like protein glycation. Further investigation of the glycation process in the course of Graves' orbitopathy is essential due to the lack of sufficient information.

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