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ORIGINAL ARTICLE

Concentration of NK cells after β-glucan and vitamin D supplementation in patients with diabetic retinopathy

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Abstract
In our study, we focused on possible effects of supplementation with glucan and vitamin D on total numbers of NK cells in patients with diabetic retinopathy. We evaluated possible relations among nutritional state (BMI), leptin levels, and total numbers of NK cells in patients supplemented with (1) glucan and vitamin D, (2) vitamin D and placebo, and (3) vitamin D alone. Our results show that 3 months of supplementation with both glucan and vitamin D resulted in significant improvements of NK cell numbers. In addition, we found statistically significant correlation between NK cell numbers and leptin levels. Based on these results, we propose that the molecule responsible for these changes is glucan, as vitamin D alone or together with placebo caused no effects.

Introduction

Diabetes mellitus type II (T2DM) is known for low secretion of insulin, glucose intolerance, and hyperglycemia and is expected to be the seventh leading cause of death in the next decade (WHO 2015). Chronic inflammations caused by long-term immunological strain, metabolic syndrome, and energetic imbalance linked with subsequent obesity are some of the most characteristic displays of T2DM (Xia et al. 2017). T2DM is a heterogenic disease lacking adequate knowledge regarding mechanisms of its induction. Among others, these mechanisms are genetical influences, which might help us to better understand this disease and its ever-growing prevalence in the world population representing significant medical, economical, and social stress (Udler et al. 2018). Whereas there are no doubts about the role of immune mechanisms in development of diabetes type I, where autoimmune mechanisms are well established (Knip and Siljander 2008). T2DM is mostly described as a metabolic problem with completely different pathogenetical observations. Lately, several cases of an involvement of chronic inflammatory reaction, including a whole spectrum of mechanisms of both nonspecific humoral and cellular response accelerated by additional influences including energetic dysbalance, have been described in T2DM (also Bonamichi and Lee 2017; Francisco et al. 2018; Lee 2014). Epidemical increase of obesity is characterized by low-grade inflammation connected with gradual growth of fat cells which are considered to be an extremely active endocrine organ producing various cytokine-like hormones (adipokines) and several pro- and anti-inflammatory factors regulating immune system (Francisco et al. 2018). T2DM is accompanied by various complications which can, during the progress of the disease, significantly support the increased damage of patient health status.

Diabetic retinopathy (DR) is one of the most common complications of T2DM with steadily increasing occurrence. Therefore, it is not surprising that evaluation of mechanisms might affect the quality of health conditions in DR patients. Searches for new ways to positively influence both induction and development of this disease are the focus of numerous laboratories (Aiello et al. 2019; Klein et al. 2008; Richter et al. 2019; Richter et al. 2018a; Richter et al. 2018b; Zavorkova et al. 2018). Our observations found not only close relations between lipid metabolism and several mechanisms of nonspecific humoral immune response, but also significant role of specific humoral immunity (Richter et al. 2018b).

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Vitamin D deficiencies were found to be associated with (1) cardiovascular problems, (2) hypertension, (3) obesity, and (4) unfavorable lipid profile. This could all possibly lead to further health complications (Jorde and Grimnes 2011). In addition, vitamin D stimulates production of some anti-inflammatory cytokines and suppresses release of pro-inflammatory cytokines. Vitamin D supplementation improved the health status of patients with high risk of diabetes development (Garbossa and Folli 2017).

We found positive effects of vitamin D supplementation, as in these patients exists a strong vitamin D deficit than in normal populations (Richter et al. 2018a). Vitamin D and β-glucan supplementation resulted in strong positive effects on general physical, mental conditions, general nutrition, lipid metabolism, leptin levels, and improvement of inflammatory status.

In the current study, we focused on evaluation of nonspecific immunity in patients with DR. Involvement of lymphocyte subpopulations (in regulation of homeostasis in a model of chronic inflammatory reaction connected with obesity and subsequent insulin resistance) was described by Olson (Olson et al. 2015). Chronic adaptive activation of immune reactions affected by elevated numbers of memory cells and reduction of numbers of $CD4^+$ cells is closely related to T2DM (Olson et al. 2015). During nonspecific cellular immune response, in individuals with a normal weight are macrophages in fat tissue only in 4% of cases. However, in the case of obese individuals, the occurrence of macrophages reached 50%. Different situations exist in NK cells, where in diabetic patients, we can find a reduction of both the total numbers and biological activity. Galactosylceramid, which is a lipid ligand, can activate NK cells and at the same time and reduce the weight. Both actions are accompanied by a decrease of low-grade inflammatory response. NK cells play decisive role in regulation of local inflammation and in prevention of metabolic syndrome, which is accompanying obesity (Xia et al. 2017). In obese patients, functional deficit of NK cells is assumed to be connected with different reaction to leptin (Laue et al. 2015). Leptin-NK cells are more cytotoxic, but longer exposure reduces these effects. Lower activity of NK cells found in our model suggests the indispensability of leptin for normal development and activation of NK cells. Some studies using a model of leptin receptor-deficient animals found that leptin is a critical regulator of both NK cell activation and development (Tian et al. 2002). The role of NK cells in T2DM was also a subject of our previous work (Pohorska et al. 2016; Richter et al. 2016).

In addition to natural killer cells (NK cells), an additional population of cells with cytotoxic abilities exist—natural killer T cells (iNKT cells). These cells represent a heterogeneous group of cells sharing properties of both NK cells and T lymphocytes and include both $NK1.1^+$, $NK1.1^-$, CD4+, $CD4^-$, $CD8^+$, and $CD8^-$ cells. In general, iNKT cells represent an evolutionary conserved population of innate-like T lymphocytes. Decrease of cytotoxic activity of NK and iNKT cells can result in higher risk of cancer development (Laue et al. 2015; Marrero et al. 2015; Parisi et al. 2017). In T2DM patients with reduced NK cell activity, there are no doubts about the protective role of both NK and iNKT cells in cancer prevention (Berrou et al. 2013; Zhang et al. 2018), particularly in induction and regulation of colorectal cancer.

In obese individuals, we can find decreased numbers of iNKT cells both in peripheral blood and omentum; bariatric surgery helps to elevate these numbers (Magalhaes et al. 2015). These authors hypothesize that microbiome alteration might be connected with clinical manifestation of T2DM, which modulates both quantity and function of both NK and MAIT (Mucosal-associated invariant cells) cells.

Despite a long history of research, the question of why to use dietary supplements remains open. The best answer is that nutrition and infection, as well as our health in general, are intimately related (Scrimshaw 2003). β-1,3-Glucan (hereafter referred to as glucan) is a natural molecule able to significantly improve our health via multifactorial biological effects. Glucan represents highly conserved structures often named pathogen-associated molecular patterns (Zipfel and Robatzek 2010). Glucans are considered to be strong activators of cellular immunity, with macrophages being the most important biological targets. Originally, glucan has been established in protection against infection. Subsequent successful studies on glucan and cancer resulted in approval of glucan as an official drug in Japan. In addition, dietary uptake of glucan is known to influence energy metabolism, probably via suppression of appetite (increase of PYY and GLP-1) and via improvements of insulin sensitivity induced by hormone secretion involving gut microbiota-produced SCFAs (Myiyamoto et al. 2018). Readers seeking the most up-to-date comprehensive review on the effects of glucan in medicine should see Vetvicka et al. (2019).

In parallel, our own research focused on children with chronic respiratory problems, followed by evaluations of its effect on development of cancer. Our studies confirmed significant improvements of salivary immunity of immunosuppressed children (Vetvicka et al. 2013; Vetvicka et al. 2019) and improvements of health status of patients with prostate cancer (Vetvicka et al. 2015). In the last decade, our laboratories conducted a series of clinical, placebo-driven trials evaluating the effects of short-term supplementation with glucan on the immune parameters in children. For these studies, we used Glucan #300, which is currently the most tested commercially available glucan with established high immunostimulating activity (Vetvicka and Vetvickova 2018). Randomly selected groups of children with chronic respiratory problems were treated with an oral dose with 100 mg of glucan per day for a period of 30 days. The results showed that this short-term supplementation significantly improved the levels of salivary immunoglobulins (Vetvicka et al. 2013), decreased

eNO levels, and improved physical endurance of children (Richter et al. 2015). Another clinical trial of the mushroom-derived glucan in myelodysplastic syndromes showed elevated functions of neutrophils and monocytes, particularly production of reactive oxygen species (Wesa et al. 2015).

The search for optimal improvement of immune reactions using various natural immunomodulators including glucan is still on. Recently, the search focused not only on potential positive regulation of cancer growth, but also on prevention of cancer (Akramiene et al. 2007; Zavorkova et al. 2018). It is important to note that moculation of defense reaction via complementary and alternative treatments was used since the dark ages (Takeda and Okumura 2004). The use of these materials is steadily increasing in the last decade as a potential step in positive regulation of the health status of general population (McFarlin et al. 2017). Glucan, either yeast- or mushroom-derived, occupies the most prominent position among immunomodulators, as demonstrated by more than 40,000 published studies.

Material and methods

Protocol

We explained the experimental protocol and obtained consent forms from all participating patients. This study was Institutional Review Board (Regional Masaryk University) approved and performed in full agreement with the Helsinki declaration (revised version 2000.09.01), and in full compliance with the Czech Republic's clinical testing rules.

Patients

A group originally consisting of 52 patients diagnosed with diabetic retinopathy complicated with diabetic macular edema in one or both eyes was divided into three groups. Nine patients were eliminated from the final results, as they did not have complete evaluation of NK cells due to absence at the final medical observation. Basic criteria were central thickness of retina above 250 μm and the best adjusted visual sharpness 4/6.3–4/40 (75 to 35 letters of EDTS phenotypes). The limiting criteria were classical laser photocoagulation of macula less than 3 months before the start of the study, previous intraocular treatment with anti-growth factor (anti-VEGF) antibodies less than 6 months before the start of the study, and situation after pars plana vitrectomy. At the beginning of the study, average thickness of retina was 448.2 ± 132.4 μm (median, 441.5) and average best adjusted visual sharpness was 63.1 ± 13.4 (median, 65.0) of ETDS letters.

Three different groups were used throughout the study. Group A was supplemented with glucan and vitamin D, group B was supplemented with vitamin D and placebo, and group C was supplemented with vitamin D only. The basic medical regime was identical in all three groups.

Glucan

Yeast-derived insoluble Glucan #300 (> 85% dry w/w basis) was purchased from Transfer Point (Columbia, SC, USA). This glucan contains 96% carbohydrates and 2.1% proteins. Neutral sugar analysis confirmed 91.3% glucose and 8% mannose. A total of 500 mg of glucan was taken on an empty stomach, followed by 100 mL of water and a 30-min rest prior to any food intake. No additional supplementation was used, including the use of aspirin. The interval of this food supplementation was 3 months. Placebo was identical in size, shape, and color. During the entire study, no side effects were observed, including any allergic reaction.

Vitamin D

Vitamin D (cholecalciferol, D3) was manufactured by Merck (Darmstadt, Germany). One milliliter of solution contains 20,000 IU of vitamin D3; one drop contains 500 IU. All patients were instructed to ingest vitamin D with fat-containing food. The dose used in our experiments was based on weight, sex, age, and phototype with 30% increase during winter months. Patients obtained all necessary information about possible risk and side effects possibly related to vitamin D supplementation (such as elevated blood levels, nausea, vomiting, stomach pain, diarrhea, or kidney failure) as recommended by the manufacturer. In all cases, we monitored possible decrease of vitamin D effects by medication (drugs containing phenytoin, rifampicin, isoniazid, barbiturates, and antacids), but no changes have been found.

Testing

Blood was collected by standard procedure after 12-h fasting at the beginning and at the end of our study. Leptin levels were determined using enzyme immunoassay (Mediagnost, Germany) with a calibrated WHO international standards EIBSC Code 97/599, which is part of the diagnostic kit.

Vitamin D levels were measured by an ELISA assay using standards recommended by the manufacturer (DRG Instruments, Germany). Based on the manufacturer's information, average values for a healthy common 58-year-old Caucasian population are 26.1 ng/mL in males and 30.2 ng/mL in females. A vitamin D deficit is considered when levels are below 10 ng/mL, insufficient levels range between 10 and 29 ng/mL, and normal levels range between 30 and 100 ng/mL. Samples were taken in the morning on an empty stomach.

NK cells were evaluated by a four-color cytometer FACS CALIBUR (BD Biosciences, USA) using anti-CD16-FITC, anti-CD56-PE, anti-CD3-PerCP, anti-CD11b-PE, and anti-

HLA-DR-PE antibodies (BD Biosciences). NK cells were $CD3^+$, $CD16^+$, $CD45^+$, and $CD56^+$. Absolute and relative levels of NK cells were calculated from the total numbers of leukocytes and lymphocytes found using a hematological analyzator XS800.

Statistical analysis

Paired *t* test statistical significance was evaluated (GraphPad Prism 5.04; GraphPad Software, USA). An average and standard deviation was evaluated after determining standard value composition (D'Agostino, Pearson). In case of nonstandard composition, values were converted into logarithms.

Results

When we monitored the length of the T2DM disease in our experimental groups, we found no statistically significant differences. Group A was the average length of T2DM 15.9 ± 7.5 years (95% CI 11.76–20.11), in group B it was 17.82 ± 9.17 years (95% CI 13.1–22.5), and in group C it was 14.0 ± 8.2 years (95% CI 8.0–19.7).

Anthropometric figures were similarly non-significant among tested groups. Average level of BMI in group A was 31.38 (95% CI 28.9–33.9), 30.57 (95% CI 27.9–33.2) in group B, and 32.1 (95% CI 28.1–36.1) in group C.

Elevated levels of leptin found in each group were also not significant. Average level of leptin in group A was 29.0 ng/mL (95% CI 12.7–45.3), 37.4 ng/mL in group B (95% CI 10.4–48.0), and 31.3 ng/mL in group C (95% CI 11.9–50.93). There is no correlation between the BMI levels and leptin levels; however, in female patients, the leptin levels are significantly higher compared with males. The average level of leptin in females was 42.8 ng/mL (95% CI 25.8–59.8); in males, it was 18.7 ng/mL (95% CI 8.02–28.33).

Leukocyte levels corresponded to increased levels of leptin. Average numbers of leukocytes in group A was 8.53×10^9/L (95% CI 6.6–10.5), 7.8×10^9/L in group B (95% CI 6.7–8.97), and 7.1×10^9/L in group C (95% CI 5.91–8.25). In group A with glucan supplementation, the number of leukocytes significantly decreased after 3-month supplementation (from 8.53×10^9/L to 7.92×10^9/L), whereas in group B and C no such trend was found. Lymphocyte numbers were not changed at all.

At the beginning of the study, the levels of NK cells were 297.1/μm (95% CI 202.0–391.0) in group A, 277.9/μm (95% CI 190.5–365.3) in group B, and 326.6/μm (95% CI 179.8–473.3) in group C. Changes in NK cell numbers are shown in Fig. 1. Group B (with placebo) showed the lowest levels of all three groups, but we assume that these levels are based on random division of patients and not on any effects of placebo used in our study. It is important to note that the differences in groups B and C were not statistically significant.

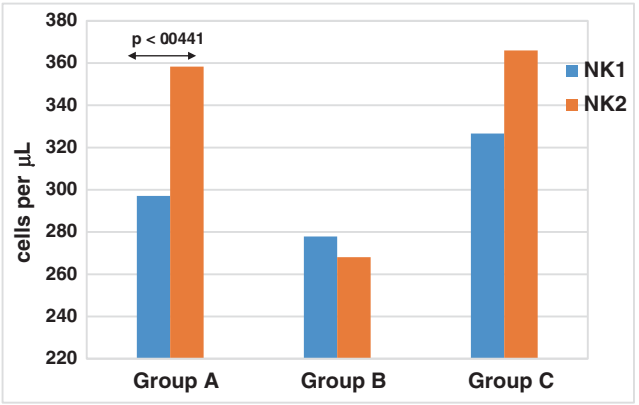

Fig. 1 Total numbers of cells before (NK1) and after (NK2) supplementation with glucan and vitamin D. Group A was supplemented with glucan and vitamin D, group B was supplemented with vitamin D and placebo, and group C was supplemented with vitamin D only. Differences in group A were statistically significant; differences in other groups were not significant

In glucan-supplemented group A, the increase was statistically significant ($p = 0.0441$); in other groups, the changes were not significant ($p = 0.376$ or $p = 0.3012$, resp.). Correlation between BMI levels and NK cell numbers was not found, but statistically significant correlation between NK cell numbers and leptin levels (linear regression 0.0442) is shown in Fig. 2. In diabetes, peripheral NK cell numbers and activities are elevated. Leptin regulates both innate and adaptive immunity and in T2DM mediates both the immune system and metabolism (Lee 2014; Cortese et al. 2019; Katsiki et al. 2018).

Discussion

Obese individuals have altered numbers and functions of NK cells. One of the possible regulators of NK cells is leptin, which directly binds to the leptin receptor present on NK cell membrane. The stimulation results in a higher production of IFN-γ. At the same type, NK cells from obese patients showed lower levels of phosphorylation in JAK2 (Laue et al. 2015). In addition, NK cell activity was found to be lower in T2DM, and it was found to be significantly related to the degree of hyperglycemia (Kim et al. 2018).

The original hypothesis about T2DM was based on the presumption of exclusive relations with malfunction of metabolic processes. However, recent findings confirmed a significant role of chronic inflammation. These chronic inflammatory processes are, among others, supported by elevated weight of patients, lipid metabolism imbalance, and changes in gastrointestinal tract microbiome. Changes in microbiome seem to be particularly interesting, as these changes might

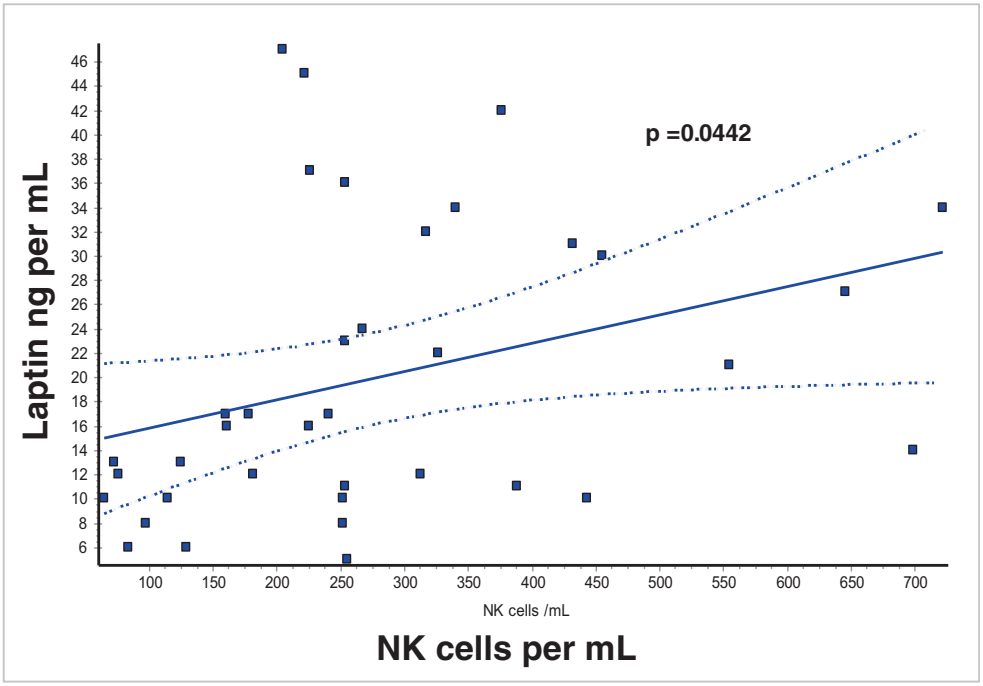

Fig. 2 Linear regression of leptin plasma levels (ng/mL) and numbers of NK cell (cells per mL) in peripheral blood at the beginning of our study

positively influence not only the initial phase of the disease, but also the entire progress of the disease (Lee 2014; Magalhaes et al. 2015; Parisi et al. 2017). Similar to the situation in T1DM patients, T2DM patients had significant defects in nonspecific cellular immune response, particularly in functions and total numbers of NK and iNKT cell population (Piatkiewicz et al. 2013). Changes in iNKT cell population in fatty tissue might start improvements of metabolic malfunctions related to regulation of obesity (Magalhaes et al. 2015). A detailed knowledge of iNKT cells, including their role in initiation of cancer development, progress of autoimmune diseases, antimicrobial immunity, and graduation of chronic inflammatory processes, would be beneficial (Coppola et al. 2015; Juno et al. 2012; Marrero et al. 2015; Pohorska et al. 2016). iNKT cells regulate functions of adipocytes and anti-inflammatory cytokine production via IL-4 and IL-10 production (Juno et al. 2012), which might be important in T2DM development. The potential role of iNKT cells in regulation of several metabolic pathways and their involvement in microbiota offer an interesting new window for possible treatment of both T1DM and T2DM (Magalhaes et al. 2015). With the effects of glucan on microbiota currently intensively studied, the potential of glucan in this area seems to be high (Jayachandran et al. 2018), but more research needs to be done.

It can be assumed that graduation of chronic inflammatory response is positively related with induction of T2DM. This hypothesis further stressed the need for detailed evaluation of the entire process of activation of adaptive immune reactions in T2DM patients (Olson et al. 2015). It would need evaluation of the wide spectrum of indicators related to not only infectious disease, but also signalizing risk on cancer disease, which in T2DM patients is very high (Berrou et al. 2013). Complex evaluation of the entire T cell population might play positive roles on both therapy and prevention of this disease (Xia et al. 2017). Knowledge of molecular mechanisms related to reparation of both quantity and quality of NK cells offers an interesting option in treatment of all these diseases (Akramiene et al. 2007; Xia et al. 2017; Yoon et al. 2015). One of the possible solutions of how to solve this problem might be identification of genetically anchored physiological mechanisms, which might help to deal with T2DM and its complications. To classify T2DM patients using genetic methods might open new doors for better diagnosis, monitoring, and treatment (Udler et al. 2018).

At the beginning of our study, we found significant decrease of numbers of NK cells, which is in agreement with findings from other laboratories (Berrou et al. 2013; Laue et al. 2015; Xia et al. 2017; Yoon et al. 2015). However, we found substantial differences among individual patients. Major differences were in patients with initiation of colorectal cancer, which was already described (Berrou et al. 2013; Coppola et al. 2015), followed by patients with inflammatory problems, particularly with urinary tract infections. Significant differences were also found in individuals with MBI type III (Berrou et al. 2013; Bonamichi and Lee 2017; Coppola et al. 2015; Juno et al. 2012; Laue et al. 2015; Marrero et al. 2015; Olson et al. 2015; Parisi et al. 2017; Xia et al. 2017). As the diet of T2DM patients is usually far from optimal, we cannot forget the possibility of nutritional effects. Both quantity and quality of our patient nutrition are typical nutrition of Czech countryside, i.e., high-energy intake

consisting of fatty pork meat and virtually no fibers reflecting motto of Czech farmers—"our pig will take the fibers for you."

Leptin is an adipokine regulating body fat mass and is one of the key factors in development of obesity via controlling the feelings of hunger (Clark et al. 2006) and by mediating the feedback in the brain related to the fat content within the body (Purve et al. 2004). In addition, it is involved in modulation of various aspects of immune responses. For a recent review of leptin and immunological profile in obesity, see Cortese et al. (2019). In mice, in prediabetic stages, total numbers of NK cell subpopulations were strongly reduced at every differentiation stage. This situation was even more influenced by significantly increased apoptosis of NK cells. Detailed studies revealed that these NK cell deficiencies were related to the leptin receptor deficiencies (Lo et al. 2007) which regulated development of NK cells in bone marrow. In addition, the synthesis of leptin was found to be regulated by glucan (Cheung 1996).

Findings of no correlation of BMI with numbers of NK cells were surprising, whereas correlation of NK cells with leptin levels was statistically significant. We assume that the use of MBI for this type of observation was not appropriate. We believe that for this relation, it might be better to use different anthropomorphic methods. Detailed evaluation of ABSI, MBI, leptin, and NK cells will be the focus of our further evaluation, which is currently in progress (Richter et al. 2018a).

Our positive experiences with glucan application in both treatment and prevention of different types of disease triggered our hypothesis about the possible supplementation with glucan for qualitative and quantitative improvements of NK cell activities in patients with diabetic retinopathy (Richter et al. 2016; Richter et al. 2018a; Richter et al. 2018b; Zavorkova et al. 2018). In addition, we based our hypothesis on data from other laboratories, describing important contributions of treatment of T2DM with the broad spectrum of different supplementation and wide evaluation of both nonspecific and adaptive immune response (Akramiene et al. 2007; Magalhaes et al. 2015; McFarlin et al. 2017; Parisi et al. 2017; Takeda and Okumura 2004; Zhang et al. 2018). However, none of these studies used supplementation with glucan, either alone or together with vitamin D, so direct comparisons are not possible. Glucan, however, is currently the most studied natural immunomodulator with at least 177 clinical trials running, making the evaluation of its effects in diabetic retinopathy even more relevant. The findings of direct correlation between leptin and NK cell levels are novel and deserve additional evaluation. Based on the results of our study, we believe that the molecule responsible for observed effect changes is glucan, as vitamin D alone or together with placebo caused no effects.

Compliance with ethical standards

This study was Institutional Review Board (Regional Masaryk University) approved and performed in full agreement with the Helsinki declaration (revised version 2000.09.01), and in full compliance with the Czech Republic's clinical testing rules.

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