Whole blood neutrophil chemiluminescence in children with diabetes depending on their clinical condition

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Abstract

The study deals with the examination of the metabolic activity of neutrophils tested with spontaneous and stimulated (fMLP, PMA, Zymosan) chemiluminescence (CL) in children with type 1 diabetes depending on their clinical conditions. We examined 87 children with type 1 diabetes from 4 to 21 years (52 girls and 35 boys, mean age – 13 years). Due to their clinical conditions, the patients were divided into three subgroups: patients in good condition (25 children), patients in mediocre condition (54 children), and patients in severe condition (8 children). The control group consisted of 23 persons – healthy volunteers from 20 to 25 years old (mean age – 23 years). We estimated the maximum levels of chemiluminescence (CLmax) with a luminometer (Microlumat LB 96P of E&G Barthold), the results were given in RLU/s. We found decreased oxide metabolism of neutrophils in patients with diabetes in all clinical conditions, both spontaneous and stimulated compared to the control group. The lowest values of neutrophil chemiluminescence were in children in mediocre condition.

Key words: *diabetes, clinical condition, neutrophil chemiluminescence, children.*

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Introduction

Type 1 diabetes develops most often in children and adolescents. Before it manifests itself, a complex process of pancreatic islets degradation develops within the pancreatic islets where both genetic and immunological factors are of crucial significance [1, 2]. Both cell-mediated and humoral mechanisms are involved in the destruction of β cells of the islets of Langerhans. This leads to autoimmune reaction as a sign of breaking tolerance to autoantigens and initiation of an immune response against one's own tissues [3, 4]. Diabetes may manifest itself many months before the process. The direct way which leads to the selective degradation of β cells in IDDM (insulin dependent diabetes) is still poorly understood. An important role in the destruction process is attributed to pro-inflammatory cytokines, especially interleukin 1 (IL-1), and nitric oxide and oxygen free radicals (RT) [4-7]. Interleukin 1 released by monocytes and macrophages and also by Langerhans cells and vascular endothelium inhibits the synthesis and release of insulin, resulting in a long-term exposure to permanent damage of β cells [8, 9]. This cytokine activates immune cells to release TNF-α and IFN-γ which then can induce neutrophils and macrophages to generate RT [10, 11]. Getting into the extracellular space, RT released from the cells damage surrounding cells, contributing to the expansion of tissue destruction and the spread of infection. The toxic activity of RT is boosted by low contents of enzymes in the cells of the islets of Langerhans (MnSOD levels in them are small even after cytokine stimulation) which decompose these highly reactive agents. The state is

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also enhanced by increased secretory activity of β cells. Metabolically active cells are much more susceptible to the damaging effects of cytokines and RT [12]. The participation of RT in the etiopathogenesis of IDDM is also confirmed by the results of tests with the use of "RT scavengers" [13]. Antioxidant compounds protect pancreatic cells from the toxic effects of cytokines [IL-1, interferon γ (IFN-γ), tumor necrosis factor (TNF)] and alloxan, while administered to animals with genetically induced diabetes retard its development [14, 15].

The aim of this study is to assess spontaneous and stimulated metabolic activity of neutrophils in the whole blood in children with type 1 diabetes depending on their clinical conditions.

Material and methods

The study included 87 children and adolescents with type 1 diabetes at the age of 4 to 21 years (52 girls and 35 boys, mean age – 13 years) treated at the Clinic of Developmental Age Endocrinology, Wroclaw Medical University. All patients received humanized insulin administered repeatedly throughout 24 hours. Information about the current course of the disease was obtained from interviews and medical records. Due to the clinical condition and degree of metabolic control of diabetes, patients were divided into three subgroups:

- good condition – 25 children (in whom acidosis occurred only once in the course of diabetes, and at the time of the study, diabetes was metabolically controlled, HbA_{1C} < 6.4%, cholesterol and triglycerides – correct values);
- mediocre condition – 54 children (acidosis occurred 2-3 times in the course of the disease, HbA_{1C} 6.5-7.5%, without glycemia and glycosuria);
- severe condition – 8 children (patients exhibit characteristics of uncontrolled metabolic diabetes, HbA_{1C} > 7.5%, frequent hyperglycemia and glycosuria, hyperlipidemia were observed; the control group consisted of 23 people – healthy volunteers aged 20 to 25 years (mean age – 23 years); they were students, because it is very difficult to obtain healthy children's blood for testing.

The study was approved by the Bioethics Committee (858/01-03) at Wroclaw Medical University.

The material for this study was venous blood, collected into plastic tubes with heparin in a closed system, while performing basic diagnostic tests or checks. The metabolic activity of neutrophils was determined with a luminol-dependent chemiluminescence test [16, 17]. Spontaneous and stimulated neutrophil CL [fMLP, opsonized Zymosan, phorbol 12-myristate 13-acetate (PMA)] was studied in 96-well, white microplate (LB96P-WMP) according to Lewkowicz *et al.* [18]. The order of addition and volume of reagents are shown in Table 1.

Each of the systems was repeated three times, and the arithmetic mean of the values received was calculated. The chemiluminescence was tested with a luminometer (Micro-Lumat LB 96P of E&G BARTHOLD). The device was controlled by WinGlow software in the Windows environment. Spot measurements were taken within 0.2 seconds every 60 seconds at 37°C. The test lasted 45 minutes. Results are expressed in RLU (Relative Light Units/s) as the maximum chemiluminescence (CL_{max}). Since CL depends linearly on the number of neutrophils, while heme compounds reduce its values, the chemiluminescence results were adjusted to their absolute number and haemoglobin content in the blood tested by the formula:

$$\frac{Hb\ [\%]}{PMN\ [\%] \times WBC\ [thous./100\ \mu l]}$$

$$CL_{CALCULATED} = CL_{DETERMINED}\ Xy$$

where Hb is haemoglobin, and PMN is the percentage of neutrophils in the total number of white blood cells – WBC.

A statistical analysis conducted using the Mann-Whitney U test involved designation of the arithmetic mean, standard deviation, and the level of significance ($p < 0.05$) for each parameter of neutrophil CL in examined patients and the control group. Using the Kruskal-Wallis ANOVA by Ranks test, box and whisker plots were made showing individual parameters of neutrophil CL including the mean, standard deviation and standard error for examined patients and the control group.

Results

Significantly lower levels of spontaneous neutrophil CL were shown in all children with diabetes (regardless of their clinical condition) compared to the control group (Fig. 1).

Table 1. The order of addition and volume of reagents

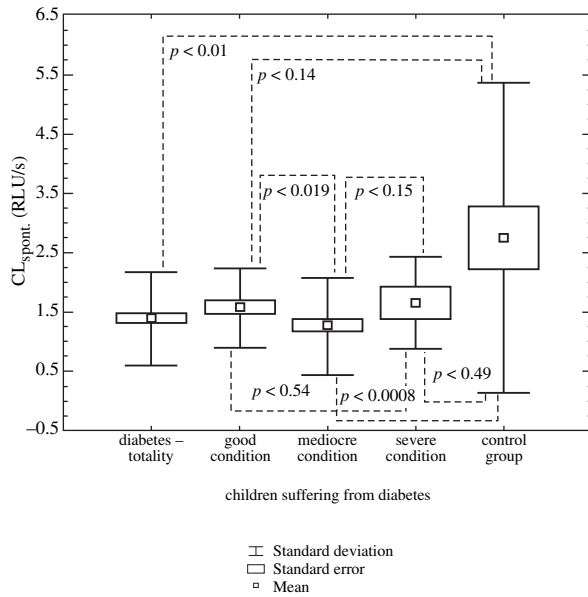| No stimulation	FMLP	PMA	Zymosan
1. PBS 40 μl	1. PBS 20 μl	1. PBS 20 μl	1. PBS 20 μl
2. Luminol 20 μl	2. fMLP 20 μl	2. PMA 20 μl	2. Zymosan 20 μl
3. Whole blood 40 μl	3. Luminol 20 μl	3. Luminol 20 μl	3. Luminol 20 μl
	4. Whole blood 40 μl	4. Whole blood 40 μl	4. Whole blood 40 μl

Fig. 1. Spontaneous chemiluminescence of neutrophils in children with diabetes compared to the control group

Fig. 2. The maximum value of neutrophil chemiluminescence after fMLP stimulation in children with diabetes compared to the control group

There were no statistically significant differences in spontaneous neutrophil CL in children in good and severe condition compared to the control group. There were statistically significant lower levels of spontaneous neutrophil CL in children in mediocre condition as compared to the control group ($p < 0.0008$), and compared to children in good condition ($p < 0.019$). The results of CL_{max} after stimulation with fMLP in the examined groups are presented in Fig. 2. There were significantly lower levels of CL_{max} in children with diabetes (all clinical conditions together) and children in mediocre condition as compared to the control group. There were no statistically significant differences in the CL_{max} levels between children in good and severe conditions, and in comparison to the control group. The results of CL_{max} after stimulation with PMA in the examined groups are shown in Fig. 3. All subgroups of children with diabetes demonstrated significantly lower CL_{max} levels compared to the control group, while the lowest values were found in children in mediocre condition. The opsonized Zymosan stimulation of neutrophils showed significantly lower CL_{max} values in all examined subgroups of children with diabetes. As in the case of fMLP and PMA stimulation, the lowest neutrophil CL levels were in children in mediocre condition.

Discussion

Diabetes is one of diseases the development of which is affected by the presence of RT. As a result of oxidative stress, elevated levels of lipid peroxidation products such as MDA, reduced level of antioxidants in the body fluids
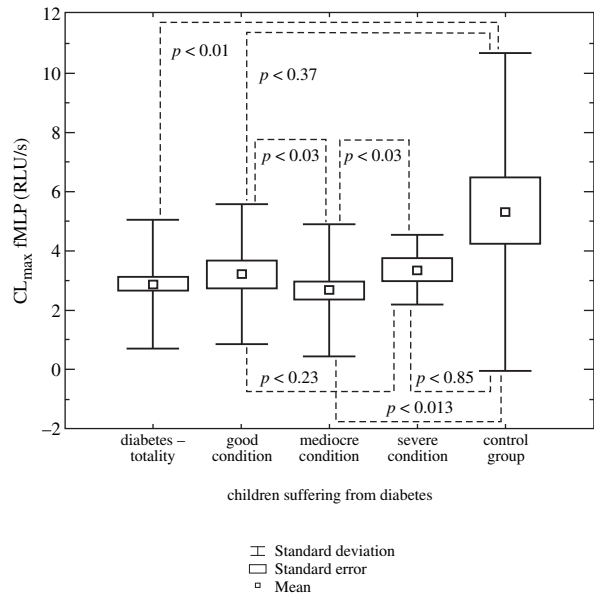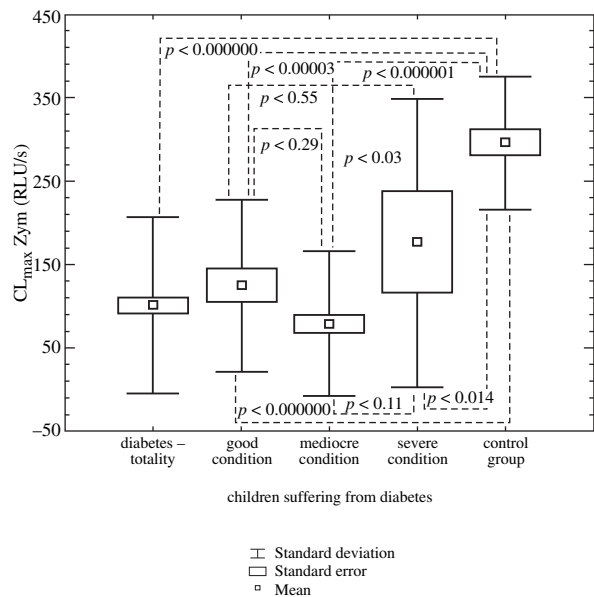

Fig. 3. The maximum value of neutrophil chemiluminescence after opsonized Zymosan stimulation in children with diabetes compared to the control group

and within cells are observed in diabetes [19]. In the early stage of type 1 diabetes in patients at developmental age, abnormalities are observed in the oxidation-reduction system, which is supported by studies conducted by Telci *et al.* [20]. They found elevated levels of markers of oxidative damage to proteins and decreased antioxidant capacity of

plasma in children with type 1 diabetes compared to the control group. In the present study we observed a decrease in neutrophil oxidative metabolism in children with diabetes compared to healthy people. We also observed impaired neutrophil response to stimulating factors, as well as a decrease of CL in the system without a stimulator (spontaneous CL) which may prove a functional "exhaustion" or "fatigue" of the cells. Studies conducted by Zgliczyński *et al.* [21] show that neutrophils may be present in different physiological states. The following neutrophils were distinguished: resting, pre-activated, activated and exhausted. Pre-activated neutrophils are in the state of functional readiness and the activation of the next stimulus causes their stronger response such as increased production of free radicals. Activated cells show a multiple increase in the release of RT [22], and are characteristic of inflammation, extensive burns and metastatic cancer [23, 24]. In diabetes, hyperglycemia may stimulate granulocytes, as in the sorbitol pathway with an increase in DAG synthesis, it activates protein kinase C – an enzyme crucial to many changes taking place in a cell [neutrophil stimulants may also be cytokines IL-1, IL-6, IL-8, TNF-α, glycated proteins (AGE)]. Their level increases in the blood of people with uncontrolled diabetes [25, 26]. Chronic neutrophil pre-activation in a high concentration of glucose in the body may lead to a decrease in the sensitivity of these cells to subsequent stimuli. This can be observed in only a slight increase in oxidative metabolism of neutrophils in response to stimuli (fMLP, PMA, Zymosan) in children with type 1 diabetes in comparison with healthy persons (the control group). Functional exhaustion of cells which results in low levels of spontaneous and stimulated chemiluminescence was also observed in other diseases such as in children or adults with recurrent respiratory tract infections [27-29]. Impaired metabolic activity of neutrophils may be caused by various factors: long-term stress, medications (antibiotics), environmental pollution, it may also be genetic. Impaired synthesis of RT by neutrophils affects the body's defence against pathogenic microorganisms, and thus its tendency to infections. It has been observed that people with diabetes are more susceptible to bacterial infection, as well as fungal and viral infections [30]. It is assumed that defects in neutrophil oxidative metabolism in children with type 1 diabetes are related, inter alia, to elevated blood glucose. In all systems tested (spontaneous CL, after stimulation with fMLP, PMA, Zymosan) significantly higher neutrophil chemiluminescence in children in good clinical condition compared to children in overall mediocre condition was reported. Slightly higher CL_{max} values were observed in children in serious condition, which disturbs the relationship. This could be due to the too small number of people in the selected group ($n = 8$) compared to children in good clinical condition ($n = 25$) and mediocre clinical condition ($n = 54$). It is worth emphasizing that all the three subgroups whose clinical conditions were taken into account, included children with both

short- and long-term diabetes, which significantly may affect the results. The presence of comorbidities, medications, blood glucose levels also affect the final results obtained. Gallacher *et al.* [31] who conducted studies on people with type 1 and type 2 diabetes, also obtained lower CL values compared to the control group. These authors also showed a negative correlation between chemiluminescence and HbA_{1C} levels in the blood. Therefore, weakening neutrophil bactericidal function should be associated not only with direct changes in blood glucose levels, but also with poor metabolic control. Another cause of impaired neutrophil oxidative metabolism may be response of these cells to insulin. Studies conducted *in vitro* by Oldenborg *et al.* [32] on healthy people showed inhibition of neutrophil chemiluminescence after stimulation with fMLP by insulin. Contrasting results were obtained by Scatena *et al.* [33] who measured opsonized Zymosan-stimulated neutrophil CL in people with diabetes, showing increased CL in comparison to the control group. Saeed *et al.* [34] showed impaired neutrophil oxidative metabolism using CL test in patients with diabetes, and they attributed the inhibitory role to acidic metabolites (acetoacetate) present in the serum of these patients. Delamaire *et al.* showed impaired neutrophil oxidative metabolism in patients with type 1 diabetes [35]. Phorbol 12-myristate 13-acetate and Zymosan-induced CL of neutrophils was lower compared to healthy people. Although a lot of studies report the participation of free radicals in the pathology of diabetes and complications typical of this disease, the present study failed to capture the moment of severe oxidative burst measured with a chemiluminescence test. Perhaps the state took place before the onset of clinical symptoms suggestive of the diagnosis of diabetes. The results of the study show decreased neutrophil oxygen metabolism in all test systems, which indicates their exhaustion. A tendency to the occurrence of recurrent infections is often observed in patients suffering from diabetes. On the one hand, the predisposing factors may include glycemic fluctuations and the resulting metabolic disorders, and, on the other hand, abnormal oxidative metabolism of neutrophils may explain the cause of the greater susceptibility of diabetic patients to infection.

The authors declare no conflict of interest.

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