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FREE RADICAL-MEDIATED DESTRUCTIVE PROCESSES IN INSULIN-DEPENDENT AND INSULIN-INDEPENDENT DIABETES MELLITUS: PROTECTIVE ACTION OF THE ANTIOXIDANT BIO-NORMALIZER

(Pilot clinical trials)

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INTRODUCTION

It has already been long known that free radicals play definite role in the development of diabetes mellitus. Thus, in 1986 Grankvist and Marklund [1] found that the isolated pancreatic β-cell membranes are the target for the attack of extracellularly generated oxygen radicals. The injury was completely suppressed by exogenous SOD and catalase but not hydroxyl radical scavengers. It has been hypothesized that oxygen radicals produced by inflamed cells may cause irreversable damage of β-cell islet in juvenile diabetes. Subsequent studies in patients and diabetic animals have provided substantial evidence for the involvement of oxygen radicals produced by the monocyte/macrophage system and neutrophils in the development of diabetes mellitus [2-6].

Free radical-mediated damage in diabetes mellitus is apparently originated from both the activation of phagocytes and the prooxidant effect of elevated glucose level. Indeed, several studies suggested that elevated glucose level in diabetic patients and experimental animals can cause membrane and lipoprotein lipid peroxidation [7-9]. An increase in lipid peroxidation and the suppression of the activities of antioxidant enzymes (erythrocyte SOD, GSH peroxidase, and catalase) have also been demonstrated [10-12]. Another possible mode of free radical damaging activity in diabetes millitus can be the reduction of NO production leading to imbalance in relaxing and contracting factors [13].

All these data as well as a certain success achieved in the treatment of diabetic animals with antioxidants and chelators [14,15] seem to us to be a relaible reason for carrying out pilot clinical study of the treatment of diabetic patients with nontoxic natural antioxidants

and chelators. Earlier, we have shown that Bio-normalizer (BN), a natural Japanese health supplementation prepared by fermentation of *Carica papya*, exhibits antioxidant and chelating properties, being an efficient modulator of free radical production by inflamed leukocytes and macrophages [16,17]. Now, we have studied the effects of short-term BN administration to the patients with insulin-dependent and insulin-independent diabetes mellitus (Type I and II) in two randomized double-blind clinical trials. The parameters of organism's free radical status before and after completing clinical trials such as oxygen radical release by monocytes and granulocytes and the activities of antioxidant enzymes were determined. In addition, the effect of BN on nitric oxide production by blood leukocytes was studied. It was found that BN was able to normalize free radical-mediated processes in diabetic patients that may to certain extent contribute to the improvement of their clinical conditions.

PATIENTS AND STUDY DESIGN

Randomized double-blind clinical trial I

30 adult patients of both sexes between 20 and 43 years with type I insulin-dependent diabetes mellitus (IDDM) took part in pilot clinical study after their informed content. Patients were given insulin at appropriate dosage, and IDDM was compensated and well controlled in all cases. Patients were divided into two groups: Group 1 of 15 patients, who were given insulin and conventional therapy, and Group 2 of 15 patients, who were given insulin, conventional therapy plus 2 sachets (6 g BN) a day for 28 days. Both groups were

similar in age, sex, body weight, the duration of IDDM and recent glycemic control. 32 health adults age-matched volunteers were used as a control group.

Randomized double-blind clinical trail II

24 adult patients of both sexes between 20 and 48 years suffered from insulin-independent diabetes mellitus (IIDM) (type II) took part in pilot clinical study after their informed content. Patients were randomized into two groups: Group 1 of 9 patients who, were given conventional sugar-decreasing therapy, and Group 2 of 15 patients, who were given conventional therapy plus 2 sachets (6 g BN) a day for 28 days. 9 adult healthy people were used as a control group.

MATERIALS AND METHODS

Lucigenin, luminol, epinephrine, 12-O-phorbol-13-myristate acetate (PMA), zymosan, N-monomethyl-L-arginine (L-NAME), Monoprep, Ficoll-Hepaque, CuZnSOD, MnSOD, and catalase were purchased from Sigma Co., St.Louis.

Monocyte and granulocyte isolation.

Mixed mononuclear cells and granulocytes from blood samples of fasting subjects were separated by density gradient cetrifugation [18]. Blood was collected into Beckton-Dickinson vacutaneers containing heparin and then layered over a Hipaque or Monoprep gradients to isolate granulocytes and monocytes, respectively. After centrifugation at 500×g for 30 min at 23°C, the bands of leukocytes were removed by aspiration, and the cells were washed twice with the ice-cold HBSS. The numbers of monocytes in

mononuclear cell population and of neutrophils in granulocyte population were verified by the Coulter Counter analysis. The cells were finally resuspended in HBSS containing mM glucose and 5% heat-inactivated calf serum. Cell suspensions were kept on ice until CL analysis.

Chemiluminescent analyses.

Lucigenin- and luminol-amplified chemiluminescence (CL) produced by monocytes or neutrophils was measured under continuous mixing on a LKB chemiluminometer (Wallach Oy, Finland at 37°C) as it has been decribed earlier [19]. Cell suspension (10^5 cells) was added into the polysterene cuvette containing 50 μM luminol or lucigenin in preheated HBSS (1 mL) and incubated for 5 min. Then, the intensity of spontaneous CL was registered continuously. After that, PMA (10 ng) or opsonized zymosan (1 mg) were added, and the CL response to stimuli was measured as the difference between the maximal intensities of stimulated and spontaneous CL.

NO production by monocytes was determined in a similar manner as a difference between luminol-amplified CL in the absence and presence of L-NAME, the inhibitor of NO-synthase.

CuZnSOD and MnSOD activities

The SOD activity in erythrocytes and neutrophils was determined by measuring lucigenin-amplified CL produced during the autoxidation of epinephrine in alkaline medium (the modified Misra and Fridovich method [20]). To prepare the SOD-containing material, washed erythrocytes or leukocytes were lysed by hypotonic shock, and

hemolysate or leukocyte lysate were added to the mixture of cold water (3.5 mL), ethanol (1.0 mL), and chloroform (0.6 mL). The mixture was vigorously shaken for 3-5 min and cetrifuged at 3000×g for 10 min. The clear top layer possessing SOD activity was immediately frozen at -20°C until performing the further analysis. Aliquot of SOD-containing extract (50 μL) was added to 0.05 M carbonate buffer (pH 9.6) containing EDTA (100 μM) and lucigenin (100 μM). The reaction was started by the addition of 50 μL epinephrine (a final concentration of 50 μM), and the CL intensity was measured continuously). The SOD activity was determined by the use of the calibration curve. MnSOD activity in leukocytes was measured in the presence of 5 mM NaCN, and CuZnSOD activity was calculated as a difference between total SOD and MnSOD activities. Protein content was determined by the Lowry method.

Statistical analysis

Dinamics of clinical and laboratory parameters before and after BN administration in both experimental and control groups was analyzed by the use of non-parametric Wilcockson criteria. Clinical and laboratory data were expressed as mean±SD. Data were analyzed statistically using nonpaired Student's test. Statistically significant difference assumed at the 5% level.

RESULTS

It was found that the production of oxygen radicals by circulating leukocytes (monocytes and neutrophils) from patients with well-controlled IDDM (the first clinical trial) was

significantly decreased as it is seen from the measurement of luminol- and lucigenin-amplified CL produced by nonstimulated, PMA- stimulated, and zymosan-monocites and neutrophils (Tables 1-4). BN-treatment of patients sharply increased practically all types of CL produced by either monocytes or neutrophils frequently make it very close to the values for normal donors. In contrast, two tendencies were observed for patients suffered from insulin-independent diabetes mellitus (Clinical trial 2): for 9 patients (Subgroup A) monocytic oxygen radical production was equal or even higher than that in control group, while 6 patients (Subgroup B) are characterized by reduced oxygen radical production. BN treatment of the patients of both subgroups normalized the radical production in both subgroups, decreasing enhanced values and increasing reduced values (Table 1).

Nitric oxide production was measured as the difference between the values of monocytic luminol-amplified CL in the absence and presence of L-NAME, an inhibitor of NO-synthase (Table 5). It is seen that BN treatment significantly enhanced NO production by monocytes of patients with IDDM.

CuZnSOD, MnSOD, and catalase activities in IDDM and IIDM circulating blood cells did not differ from those in donor cells, and BN treatment did not affect the initial levels of the activities of antioxidant enzymes (data not shown).

DISCUSSION

Our findings suggest that oxidative metabolism of leukocytes (monocytes and neutrophils) indeed significantly affected in the patients with diabetes mellitus. To our surprise, we found drastic difference in the oxidative metabolism of leukocytes from patients with

insulin-dependent and insulin-independent diabetes mellitus: the oxygen radical production by IDDM cells was sharply suppressed in comparison with normal donors, while patients with IIDM must be divided into two subgroups with enhanced and reduced monocytic production of oxygen radicals. Our data for IDDM patients do not contradict the results obtained earleir for the patients with pronounced but not newly diagnosed IDDM [3, 21] because the initial stage of diabetes mellitus is apparently always characterized by the enhanced level of oxygen radical production by leukocytes. However, insulin is an inhibitor of oxygen radical release by leukocytes [21], therefore its prolonged administration to patients may be a major reason of the reduced oxygen radical release by leukocytes.

In accord with the above proposal, leukocytes from IIDM patients should produce the enhanced level of oxygen radicals. It seems to be the case for 9 of 15 patients with insulin-independent diabetes mellitius although oxygen radical production by monocytes of 6 other patients was not enhanced or even slightly decreased (Table 1). The reason of that remains unclear.

The impaired oxygen metabolism of circulating phagocytes can be an origin of the enhanced susceptibility of diabetic patients to infections and may at least partly explain numerous immune abnormalities also frequently observed in these patients. Therefore, we believe that the application of Bio-normalizer capable of normalizing the oxygen radical production by inflamatory cells [16,17] is a perspective route for combating these toxic manifestations. It was found that BN administration indeed brings the enhanced and reduced levels of oxygen radica production by both IDDM and IIDM cells to normal values. (It should be stressed that there were no any significant changes in the leukocyte

oxidative metabolism of patients of control groups). It seems to be very important that monocytic NO production was also normalized after BN administration that can induce improvement in vascular permeability and tonus and lead to fast wound healing.

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Table 1

Luminol-amplified CL produced by blood monocytes of control and BN-treated patients (m ± SD)

Group	Spont. CL before	Spont. CL after	PMA-stim. CL before	PMA-stim. CL after	Zymosan-stim. CL before	Zymosan-stim. CL after
Control, (trial 1)	56±40	74±60	220±100	320±190	300±200	560±310
BN-treated (trial 1)	100±80	240±100	460±220	840±340	520±400	3350±160
Control, (trial 2)	1200±450	650±500			8050±650	15500± 6200

BN-treated (trial 2). subgroup 1	1020±220	615±360			21100± 8400	7100± 1970
BN-treated (trial 2), subgroup 2	100±60	530±250			1720±640	3800±960
Normal donors	520±210				4220±2500	

Table 2

Lucigenin-amplified CL produced by blood monocytes of control and BN-treated patients (m ± SD)

Group	Spont. CL before	Spont. CL after	PMA-stim. CL before	PMA-stim. CL after	Zymosan-stim. CL before	Zymosan-stim. CL after
Control, (trial 1)	13±10	14±10	45±25	43±19	30±20	51±30
BN-treated (trial 1)	6±3	12±7	40±30	120±80	90±80	810±450

Table 3

Luminol-amplified CL produced by blood neutrophils of control and BN-treated patients (m ± SD)

Group	Spont. CL before	Spont. CL after	PMA-stim. CL before	PMA-stim. CL after	Zymosan-stim. CL before	Zymosan-stim. CL after
Control, (trial 1)	190±130	190±130	990±470	770±500	3370± 2380	1650± 1500
BN-treated	210±180	920±470	1270±	2320±	3140±	15500±

(trial 1)			1050	1700	2500	7700

Table 4

Lucigenin-amplified CL produced by blood neutrophils of control and BN-treated patients (m ± SD)

Group	Spont. CL before	Spont. CL after	PMA-stim. CL before	PMA-stim. CL after	Zymosan-stim. CL before	Zymosan-stim. CL after
Control, (trial 1)	35±17	23±11	98±59	103±80	210±160	135±110
BN-treated (trial 1)	27±18	49±25	75±50	260±150	390±300	2100±700

Table 5

L-NAME-inhibited luminol-amplified CL produced by monocytes of control and BN-treated patients (m ± SD)

Group	PMA-(PMA+L-NAME)		Zymosan - (Zymosan+L-NAME)	
	before	after	before	after
Control, (trial 1)	160	130	110	180
BN-treated (trial 1)	290	480	160	1480