Increase in glycaemia stimulates reactive oxygen species (ROS) production by polymorphonuclear neutrophils in type 2 diabetic patients

Dorota Pisarczyk-Wiza1, Dorota Zozulińska-Ziółkiewicz1, Henryk Wysocki2, Bogna Wierusz-Wysocka1

1 Department of Internal Medicine and Diabetology, University of Medical Sciences, Poznan, Poland
2 Department of Cardiology and Intensive Therapy, University of Medical Sciences, Poznan, Poland

Abstract

Polymorphonuclear neutrophils (PMNs), oxidative stress and hyperglycaemia play important roles in the development of micro- and macroangiopathy in diabetes. The aim of this study was to evaluate the influence of a meal and the increment of postprandial glycaemia on reactive oxygen species production by PMNs in type 2 diabetic patients. The study was performed on 25 patients – 18 women and 7 men, aged 61.7±10.5 years, diagnosed with type 2 diabetes for 16.0±7.5 years, and in 20 healthy control subjects. PMNs were isolated from the blood by single-step gradient centrifugation. The superoxide anion (O2–) production by PMNs was measured spectrophotometrically by cytochrome C reduction, and the hydrogen peroxide (H2O2) level was measured spectrophotometrically by phenyl red oxidation. A significant correlation was observed between an increase in glycaemia and O2– production (r=0.52, p<0.05) and in H2O2 production by PMNs (r=0.43, p<0.05). The results obtained suggest that in obese type 2 diabetic patients it is the increment of postprandial glycaemia, and not directly the meal itself, that stimulates reactive oxygen species production by PMNs. This might play an important role in the development of late vascular complications and thus have clinical implications.

Key words

reactive oxygen species, polymorphonuclear neutrophils, postprandial state, diabetes mellitus type 2

INTRODUCTION

Chronic complications of diabetes continue to create a significant clinical problem. Macroangiopathy, microangiopathy and diabetic neuropathy lead to severe disability and premature death. To date, the responsible pathogenetic mechanisms have not been fully understood; nevertheless, hyperglycaemia is known to involve a factor which plays a key role in the induction and progression of chronic diabetic complications. This was proved in the Diabetes Control and Complications Trials (DCCT) studies performed on type 1 diabetic patients, as well as in both the Kumamoto Study and the United Kingdom Prospective Diabetes Study (UKPDS) which involved patients with type 2 diabetes [1-3]. All these studies clearly demonstrated that efforts to reach normoglycaemia and optimal metabolic control significantly reduce the complications of diabetes, especially those involving microangiopathy. In recent years attention has been drawn to the role played by abnormal postprandial glycaemia. Its role in the development of atherosclerotic lesions was observed by Hanefeld et al. [4]. Their results indicate that postprandial hyperglycaemia represents an independent risk factor of death in patients with newly diagnosed diabetes. In addition, several other, earlier reports, including the Honolulu Heart Study, the Whitehall Study, Paris Prospective Study and the Helsinki Policemen’s Study, all showed that even a slight increase in the blood glucose level following a meal significantly increased the risk of cardiovascular disease [5-7]. Ceriello et al. have suggested that postprandial increases in the blood glucose concentration may lead to abnormalities in the function and structure of cells in the vascular wall [8]. Together with others, Ceriello et al. noticed that hyperglycaemia may be responsible for the pronounced apoptosis of these cells due to intensified protein glycation, increased production of reactive oxygen species, and alterations in the clotting and fibrinolysis mechanisms through the activation of protein kinase C, and of the transcription nuclear factor NFκ-B [8-10].

In recent years, the inflammatory theory of the development of chronic diabetic complications has gained an increasing number of adherents [11-13]. The syndrome of hyperglycaemia-induced disturbances corresponds to lesions which can be detected at inflammatory foci. The principal cells involved in a non-specific inflammatory response are polymorphonuclear neutrophils (PMNs). These circulate in the blood for only 6–8 hours at rest, but in a state of readiness they become stimulated. If not stimulated, i.e. in normal conditions, a significant proportion of PMNs undergo apoptosis. The remaining fraction leaves the circulation and undergoes destruction in the tissues, or on the surface of mucous membranes. Activated PMNs alter their properties and adjust to chemotaxis and to interaction with themselves (aggregation) and to other cells, such as monocytes, blood platelets, endothelial cells (adhesion). The PMN activation stage involves diapedesis and migration of cells through the
endothelium. They reach the sites at which inflammation- 
inducing factors are acting and there phagocytose the foreign 
bodyes. The process of degradation of phagocytised material 
requires the oxygen species (ROS) and proteolytic 
enzymes released into the phagosome. The condition in which 
increased activities of reactive oxygen species are detected 
used to be termed ‘oxidative stress’ [14,15]. The stress develops 
due to disturbed equilibrium between ROS production and 
the capacity to eliminate them by anti-oxidative systems. The 
reactive oxygen species include, among others, superoxide 
anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical 
(·OH), hydrogen superoxide (HO$_2$), single oxygen (O$_3$) and 
nitric oxide (NO) [14,16,17]. ROS may manifest their toxic 
activity not only within a phagosome but also outside the cell. 
The phenomenon develops in the presence of chemotactic factors. 
Mediators of the inflammatory response, released from 
activated PMNs, induce destructive processes which 
affect the neighbouring tissues.

Apart from the action of classical inflammatory agents, 
activation of PMN may also result from the action of metabolic 
Factors. In conditions of hyperglycaemia the cells manifest 
permanent activation and therefore a reduced response to 
standard stimulatory agents [18-20]. It was suggested recently 
that the intensity of the atherogenic inflammatory process may 
reflect, among other factors, superphysiological increments in 
glycaemia. We therefore decided to determine the effect of a 
meal on reactive oxygen species release from PMNs.

**MATERIALS AND METHODS**

Evaluation of the influence of ingesting a standard meal 
on ROS production by PMNs was performed on 25 patients 
with type 2 diabetes – including 18 women and 7 men, 44-
74 years of age, with a history of clinically evident diabetes 
and the values of glycaemia and the 
frequency of reactive oxygen species release from PMNs.

**Table 1. Clinical characteristics of patients with type 2 diabetes and controls**

<table>
<thead>
<tr>
<th>Description</th>
<th>Controls (n=20)</th>
<th>Diabetics (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Gender (females/males)</td>
<td>18/7</td>
<td>10/10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.7 ± 10.5</td>
<td>30.0 ± 8.9</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>16.0 ± 7.5</td>
<td>-</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>157.4±51.5 [8.7±2.8]</td>
<td>70.3±10.1 [3.8±0.6]</td>
</tr>
<tr>
<td>Glycaemia 2 hours after a meal (mg/dl)</td>
<td>229.5±77.9 [12.7±4.3]</td>
<td>74.4±8.0 [4.1±0.4]</td>
</tr>
<tr>
<td>Increment of glycaemia (mg/dl)</td>
<td>-</td>
<td>72.1±64.7 [4.0±3.9]</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.4±1.9</td>
<td>5.1±1.8</td>
</tr>
<tr>
<td>Fructosamine (μmol/l)</td>
<td>385.6±67.8</td>
<td>187.2±27.8</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl) [mmol/l]</td>
<td>232.2±38.0 [6.0±1.0]</td>
<td>181.7±34.8 [4.7±0.9]</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl) [mmol/l]</td>
<td>41.6±19.7 [1.1±0.5]</td>
<td>46.4±11.6 [1.2±0.3]</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl) [mmol/l]</td>
<td>142.6±39.0 [3.7±1.0]</td>
<td>92.8±11.6 [2.4±0.3]</td>
</tr>
<tr>
<td>Triglycerides (mg/dl) [mmol/l]</td>
<td>267.7±170.8 [3.0±1.9]</td>
<td>106.3±35.4 [1.2±0.4]</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.9±6.3</td>
<td>22.5±1.2</td>
</tr>
<tr>
<td>WHR</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>146±14</td>
<td>116±18</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>85±10</td>
<td>68±10</td>
</tr>
<tr>
<td>WBC level upon fasting (×10⁹/l)</td>
<td>5.6±1.4</td>
<td>6.3±0.4</td>
</tr>
<tr>
<td>WBC level 2 hours after a meal (×10⁹/l)</td>
<td>6.0±1.9</td>
<td>7.1±0.6</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>6.4±4.8</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>HOMA-IR (%)</td>
<td>13.0±2.8</td>
<td>-</td>
</tr>
<tr>
<td>HOMA-B (%)</td>
<td>158.6±29.9</td>
<td>-</td>
</tr>
<tr>
<td>Microalbuminuria (n)</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>Retinopathy (n)</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>Neuropathy (n)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Stroke (n)</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Ischaemic cardiac disease (n)</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Insulin alone (n)</td>
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</tr>
<tr>
<td>Insulin + metformin (n)</td>
<td>6</td>
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</tr>
<tr>
<td>Metformin (n)</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Metformin + Sulfonylurea (n)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Diet only (n)</td>
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<td>-</td>
</tr>
<tr>
<td>ACE-inhibitors (n)</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>Statins (n)</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Fibrates (n)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Acetylsalicylic acid (n)</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>Indapamide (n)</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Calcium channel antagonists (n)</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Beta-blockers (n)</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

HOMA-IR = insulinaemia upon fasting (μU/ml) × 
glycaemia upon fasting (mmol/l)/ 22.5. 
HOMA-B = insulinaemia upon fasting (μU/ml) × 
20/ [glycaemia upon fasting (mmol/l) – 3.5].

Studies were performed in all the diabetic patients to detect 
any chronic complications of the disease. Retinopathy was 
diagnosed by examination of the fundus of the eyes after
dilatation of the pupils. Nephropathy was diagnosed by the presence of microalbuminuria (2 positive results of 3 tests). The presence of any neuropathy was diagnosed on the basis of the history, tests of the Achilles tendon reflex, sense of touch and vibration. The presence of cardiovascular disease was detected via the history, 12-lead ECG examination, echocardiography and evaluation of the pulse in the dorsal arteries of the feet.

In all subjects the parameters of metabolic control (fasting plasma glucose level, postprandial plasma glucose level, serum fructosamine level, serum concentration of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) were evaluated using a standard technique and the HbA1c level by means of high liquid chromatography (HPLC).

The white blood cell (WBC) count in the fasting state, and 2 hours after a meal, were assessed using standard laboratory techniques. The serum insulin concentration was measured using the immunoenzymatic technique, and C-reactive protein by the highly sensitive immunoturbidimetric technique using HITACHI 717 kits.

For studies on the function of PMNs, 6 ml of peripheral blood was taken from a cubital vein into plastic tubes containing heparin to a final concentration of 1 unit/ml blood. The blood was sampled twice: before and 2 hours after a standard meal rich in carbohydrates, containing 30 g (126 kcal or 60%) carbohydrates, 6 g (54 kcal or 25%) fat and 8 g (34 kcal or 15%) protein. The total calorific value of the meal amounted to approximately 214 kcal. The studies were performed on PMNs isolated from the venous blood.

Isolation of PMNs according to a modified version of the Boyum technique [21]. Six ml of blood was overlaid on 3 ml of Gradisol G preparation and centrifuged for 30 min at 2,000 rpm. After collection of the PMN-rich buffy coat, the cells were washed twice with buffered saline. The remaining admixture of erythrocytes in the sediment was lysed using a hypotonic lysing agent (a reagent containing 0.828 g NH₄Cl, 0.1 g KHCO₃ dissolved in 100 ml distilled water, with the addition of 20 μl 1 N NaOH). The isolated PMN were suspended in a modified Hanks solution (MHS) (11 mM glucose, 9 mM KH₂PO₄, 145 mM NaCl, 1 mM HCl, pH 7.4), in the volume necessary to recover 70%-90% of the original cell content. The final cell concentration appropriate for the planned experiments was 5×10⁶ cells/ml. Using this technique it was possible to recover 70%-90% of the original cell content. The final cell concentration was performed on PMNs isolated from the venous blood.

Production of hydrogen peroxide (H₂O₂) by PMNs according to the modified technique of Pick [23]. Each of the 96 wells in a microtitration plate was charged with 25 μl of the suspension of the PMN (5×10⁶ cells/ml) and with 50 μl of phenol red solution. Subsequently, 25 μl MHS was added to each well. Following incubation (30 min., temp. 37°C), the reaction was stopped by the addition of 10 μl 1 N NaOH. The amount of H₂O₂ was evaluated spectrophotometrically at the wave length of λ=600 nm, using a standard curve, drawn every time for a row of respective hydrogen peroxide dilutions. The results were expressed in nmol/5×10⁶ PMNs/30 min.

Statistical evaluation of the results. Normality of distribution was tested using the Kolmogorov-Smirnov test.

In cases of normal distribution in the results of the effect of a meal on PMN function, Student’s t test was used. When the results manifested any distribution that differed from the normal, the Wilcoxon’s non-parametric test was used.

Differences between the groups being evaluated were appraised using the Mann-Whitney test. The relationship between increases in glycaemia and reactive oxygen species production by PMNs was assessed using the Pearson (r) correlation test. Values at p<0.05 were accepted as representing significant differences.

The results of O₂⁻ and H₂O₂ production by PMNs were presented as means ± standard error of the mean (SEM).

RESULTS

In the group of healthy individuals, fasting glycaemia averaged 70.3±10.1 mg/dl (3.8±0.6 mmol/l); 2 hours after the meal it was 74.4±8.0 mg/dl (4.1±0.4 mmol/l). No significant postprandial increase in glycaemia was found (p>0.05).

In the patients with type 2 diabetes, fasting glycaemia averaged 157.4±51.5 mg/dl (8.7±2.8 mmol/l), this being significantly higher than in healthy individuals (p<0.05). Two hours after the meal, glycaemia increased significantly to a mean of 229.5±77.9 mg/dl (12.7±4.3 mmol/l) (p<0.05). In all the type 2 diabetic patients the meal induced an increase in glycaemia by a mean of 72.1±64.7 mg/dl (4.1±3.9 mmol/l).

In the healthy subjects, the mean WBC level before the standard meal was 6.3±0.4 (×10⁹/l), and following the meal it rose to 7.1±0.6 (×10⁹/l). In the type 2 diabetic patients, mean WBC values were similar being, respectively, before the meal: 5.6±1.4 (×10⁹/l) and following the meal 6.0±1.9 (×10⁹/l).

Effect of the test meal and the postprandial increase in glycaemia on the production of superoxide anions (O₂⁻) by PMNs (Figs. 1 and 2). Production of O₂⁻ upon fasting was significantly higher in the type 2 diabetic patients than in the healthy individuals (8.40±1.11 nmol/5×10⁶PMNs/30 min vs. 4.28±0.59 nmol/5×10⁶PMNs/30 min, p<0.01). On the other hand, 2 hours after the meal, no significant differences in O₂⁻ production could be detected between the 2 groups.
after the meal

In the healthy individuals, a significant increase in O$_2^-$ production was detected 2 hours after the standard meal (4.28±0.59 nmol/5x10^6 PMNs/30min vs. 10.54±2.65 nmol/5x10^6 PMNs/30min, p>0.05). In these patients, the production of H$_2$O$_2$ by PMNs (10.54±2.65 nmol/5x10^6 PMNs/30min vs. 7.10±1.14 nmol/5x10^6 PMNs/30min, p>0.05) or 2 hours after the meal failed to significantly affect the production of O$_2^-$ in type 2 diabetic patients (8.40±1.11 nmol/5x10^6 PMNs/30min vs. 7.10±1.14 nmol/5x10^6 PMNs/30min, p>0.05). However, in the diabetic patients, the production of O$_2^-$ by PMNs in the second hour after the meal demonstrated a significant correlation with the postprandial increase in glycaemia (r = 0.52, p<0.05). No correlation was found between the postprandial increase in glycaemia and O$_2^-$ production by PMNs in the healthy controls (p>0.05).

**Effect of the test meal and postprandial increase in glycaemia on the production of hydrogen peroxide (H$_2$O$_2$) by PMNs (Figs. 3 and 4).** No significant differences were discovered between the examined groups in the production of H$_2$O$_2$ by PMNs upon fasting (20.68±1.20 nmol/5x10^6 PMNs/30min vs. 21.99±1.24 nmol/5x10^6 PMNs/30min, p>0.05). However, in the diabetic patients, the production of H$_2$O$_2$ by PMNs in the type 2 diabetes control group (24.01±1.68 nmol/5x10^6 PMNs/30min vs. 21.99±1.24 nmol/5x10^6 PMNs/30min, p>0.05) or 2 hours after the meal was found to exhibit a significant correlation with the postprandial increase in glycaemia (r = 0.44, p<0.05). No such correlation was found between the postprandial increase in glycaemia and H$_2$O$_2$ production by PMNs in the healthy controls (p>0.05).

**DISCUSSION**

Polymorphonuclear neutrophils appear to play a significant role in the development of diabetic micro- and macroangiopathy. As cells of the inflammatory response they are engaged in processes induced by hyperglycaemia, leading to injury of the vascular endothelium. In diabetic patients, several disturbances of PMN function were noticed [24-27]. Wierusz-Wysoczka et al., demonstrated negative effects of increasing glucose concentrations on some PMN functions in *in vitro* conditions [19,20]. PMNs are a significant source of reactive oxygen species, providing basic elements for the oxygen-dependent bactericidal mechanisms. Disturbance in the properties (e.g., an inborn deficit in NAPDH oxidase) may lead to severe, frequently lethal infections [28]. An increase in ROS production by PMNs may result from humoral factors, including an acute or chronic hyperglycaemia [29]. Most reports in the literature draw attention to increases in ROS production by unstimulated PMN and the less effective response to activating factors in diabetic patients [30]. This has been confirmed by the results of our study which showed that the production of O$_2^-$ upon fasting was significantly higher in patients with type 2 diabetes than in normal individuals. Ceriello et al. observed a relationship between the development of oxidative stress and rapid, postprandial increments in glycaemia. These processes include, among others, the stimulated glycation of proteins, auto-oxidation of glucose, and the intracellular activation of glucose turnover along the polyol pathway [8,9]. In our
study, glycaemia increased following the meal, but no parallel increase was observed in the production of superoxide anions or hydrogen peroxide by PMNs isolated from the blood of patients with type 2 diabetes. The absence of the effects of a on the production of ROS by PMNs in diabetics may point to a disturbed reactivity of the cells to additional stimulatory factors. In patients with type 2 diabetes, PMNs and, most probably, their precursors in the bone marrow, are exposed in a chronic fashion to elevated glucose concentrations. Poor metabolic control was manifested by the abnormal, elevated levels of fructosamine and Hba1c at the beginning of the studies. Zozulińska et al. have already shown that PMN function is dependent on the Hba1c value [31]. The additional stimulatory factors might also have involved the hyperlipemia and hypertriglyceridemia, in particular, which we also noted in our studies. Under such conditions, the PMNs probably remain in a permanent state of sub-threshold stimulation. Several authors have suggested that PMNs in this state are unable to respond appropriately to the action of typical stimulatory factors [25,32-34]. The standard meal applied in our studies apparently failed to provide a sufficiently strong stimulus to activate the sub-threshold activated cells. However, attention should be drawn to the significant relationship noted in our studies between the postprandial increment in glycaemia and the production of O$_2^-$ and H$_2$O$_2$ by PMNs in patients with type 2 diabetes 2 hours after the meal. The relationship demonstrated is consistent with the suggestions of Ceriello et al., that the augmented ROS production is primarily responsible for the postprandial ‘toxicity’ of hyperglycaemia (35). Our studies additionally draw attention to the potential role of PMNs in the initiation of the acute process of postprandial damage to vascular walls.

In contrast to the diabetic patients, in healthy individuals the meal exerted a significant effect on the intensity of O$_2^-$ and H$_2$O$_2$ production by isolated PMNs. These results are consistent with the work of Mohanty et al., who showed that loading with 75g glucose was followed in the subsequent 3 hours by increased production of ROS by PMNs and monocytes isolated from the blood of healthy individuals [29]. The most pronounced production of reactive oxygen species was recorded in the second hour of the test. They demonstrated an increased release of ROS by PMNs and monocytes obtained from healthy individuals following a meal rich in lipids and proteins [36]. Their results, as well as those obtained in our study, suggest that the function of PMNs in healthy individuals may be dependent on the composition of meals, including the carbohydrate content. The response of PMN to alimentary stimulus shows how cells involved in the inflammatory response behave under physiological conditions. PMNs released from the marginal pool under the effect of a meal remain in the pre-activation phase. In this state, they are characterized by high sensitivity to the action of sequential stimulatory factors and are capable of releasing reactive oxygen species. In the PMNs of healthy individuals, the augmented production of ROS follows the intensity of the intracellular glucose metabolism. Loading with glucose directly activates NADPH oxidase in PMN, the key enzyme for the production of superoxide anions. In turn, the superoxide anion provides a substrate for the cascade generation of ROS [14,15,37]. In physiological conditions, reactive oxygen species exert no particularly destructive effects on tissues: their damaging affect on cells is counteracted by effective anti-oxidative systems. Ceriello et al. demonstrated that in the course of an oral glucose tolerance test performed on healthy individuals, the increment in blood glucose concentration is paralleled by an increase in blood malonyldialdehyde (MDA) concentration, and by a decrease in the total anti-oxidative capacity of plasma. Apparently, in patients with metabolically unbalanced diabetes, anti-oxidative systems exhibit low efficacy.

The role of PMNs in the development of atherosclerotic lesions seems to be increasingly well documented. Postprandial hyperglycaemia and hyperlipaemia modulate the course of the inflammatory response, including the functions of PMNs. We found that a meal rich in carbohydrates modulates the functions of the PMN cells, and the effect in patients with type 2 diabetes was different from that in normal individuals. Determination of the role of various other factors on the functions of PMNs requires further study.

**ABBREVIATIONS**

- O$_2^-$ = superoxide anion
- BMI = Body Mass Index
- BP = Blood Pressure
- CRP = C-reactive protein
- DCCT = Diabetes Control and Complications Trials
- H$_2$O$_2$ = hydrogen peroxide
- Hba1c = glycated haemoglobin
- HDL = high density lipoproteins
- HMPS = hexomonophosphate pathway
- HO$_2$ = hydrogen superoxide
- HOMA = Homeostasis model assessment
- HOMA-B = Homeostasis Model Assessment-beta cells function
- HOMA-IR = Homeostasis Model Assessment-insulin resistance
- LDL = low density lipoproteins
- MDA = malonyldialdehyde
- MHS = modified Hanks solution
- NFκ-B = transcription nuclear factor kappa-B
- NO = nitric oxide
- O$_2$ = superoxide anion
- OH = hydroxy radicals
- PKC = protein kinase C
- PMN = polymorphonuclear neutrophils
- ROS = reactive oxygen species
- SOD = superoxide dismutase
- TG = triglycerides
- UKPDS = United Kingdom Prospective Diabetes Study
- WBC = white blood cell
- WHR = Waist to Hip Ratio

**AUTHORSHIP RESPONSIBILITY**

Dorota Pisarczyk-Wiza (DPW) contributed to the performance of the study and writing article. Dorota Zozulinska-Ziolkiewicz (DZZ) and Bogna Wierusz-Wysoka (BWW) contributed to the conception and design of the study. Dorota Zozulinska-Ziolkiewicz additionally carried out the statistical analysis of the data. Henryk Wysocki (HW) cooperated in the performance of the study.
REFERENCES


