Impact of intensive insulin treatment on the development and consequences of oxidative stress in insulin-dependent diabetes mellitus

Uticaj intenziviranog lečenja insulinom na razvoj oksidativnog stresa i njegove posledice kod insulin-zavisnog dijabetes melitusa

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Abstract

Background/Aim. The aim of this study, which included patients with insulin-dependent diabetes mellitus, was to determine the influence of the application of various treatment modalities (intensive or conventional) on the total plasma antioxidative capacity and lipid peroxidation intensity expressed as malondialdehyde (MDA) level, catalase and xanthine oxidase activity, erythrocyte glutatione reduced concentration (GSH RBC), erythrocyte MDA level (MDA RBC), as well as susceptibility of erythrocyte to H_2O_2-induced oxidative stress. **Methods.** This study included 42 patients with insulin-dependent diabetes mellitus. In 24 of the patients intensive insulin treatment was applied using the model of short-acting insulin in each meal and medium-acting insulin before going to bed, while in 18 of the patients conventional insulin treatment was applied in two (morning and evening) doses. In the examined patients no presence of diabetes mellitus complications was recorded. The control group included 20 healthy adults out of a blood doner group. The plasma and erythrocytes taken from the blood samples were analyzed immediately. **Results.** This investigation proved that the application of intensive insulin treatment regime significantly improves total antioxidative plasma capacity as compared to the application of conventional therapy regime. The obtained results showed that the both plasma and lipoproteines apo B MDA increased significantly more in the patients on conventional therapy than in the patients on intensive insulin therapy, most probably due to intensified xanthine oxidase activity. The level of the MDA in fresh erythrocytes did not differ significantly between the groups on intensive and conventional therapy. The level of GSH and catalase activity, however, were significantly reduced in the patients on conventional therapy due to the increased susceptibility to H_2O_2-induced oxidative stress . **Conclusion.** The presented study confirmed positive effect of intensive insulin therapy on metabolic control expressed through glycemia level, glycolysed hemoglobine (HbAlc) and fructosamine, as well as through antioxidative/prooxidative homeostasis. This is the confirmation that an adequate treatment choice can prevent numerous diabetes mellitus complications induced by free radicals.

Key words:
diabetes mellitus, type 1; hypoglycemic agents; insulin; free radicals; lipid peroxidation; plasma; erythrocytes; oxidative stress.

Apstrakt

Uvod/Cilj. Ova studija, kojom su obuhvaćeni bolesnici sa insulin-zavisnim dijabetesom, imala je za cilj da utvrdi uticaj primene različitih modaliteta (intenziviranog ili konvencionalnog) lečenja insulinom na ukupni antioksidativni kapacitet i intenzitet peroksidacije lipida, izražen kao nivo malondialdehida (MDA) plazme, aktivnost katalaze i ksantin oksidaze, koncentraciju redukovanog glutationa eritrocita (GSH RBC), nivo peroksidacije lipida u eritrocitima (MDA RBC), kao i na podložnost eritrocita oksidativnom stresu u prisustvu H_2O_2. **Metode.** U studiju je bilo uključeno ukupno 42 bolesnika sa insulin-zavisnim dijabetesom. Kod 24 bolesnika primenjeno je intenzivirano lečenje insulinom po modelu primene kratkodelujućih insulina uz svaki obrok i srednjedelujućih insulina pred spavanje. Kod 18 bolesnika primenjeno je konvencionalno lečenje insulinom u dve (jutartnja i večernja) doze. Kod ispitivanih bolesnika nije registrovano prisustvo komplikacija dijabetes melitusa. Kontrolnu grupu činilo je 20 zdravih odraslih osoba odabranih među dobrovoljnim davaocima krvi. Plazma i eritrociti dobijeni iz uzoraka krvi ispitanika odmah su prosleđivani na analizu. **Rezultati.** Istraživanjem je dokazano da primena intenziviranog režima terapije insulinom značajno popravlja ukupni antioksidativni kapacitet plazme u odnosu na primenu konvencionalnog režima terapije. Dobijeni rezultati pokazali su da je MDA plazme i apo B lipoproteina porastao značajno više kod bolesnika na konvencijalnoj terapiji nego kod bolesnika na intenziviranoj terapiji insulinom, najverovatnije usled pojačane aktivnosti ksantin oksidaze. Nivo MDA u svežim eritrocitima nije se značajno razlikovao u grupama na intenzivnoj ili konvencionalnoj terapiji. Nivo GSH i aktivnost katalaze, međutim, bili su značajno niži kod bolesnika na konvencionalnoj terapiji usled povećane podložnosti oksidativnom stresu u prisustvu H_2O_2. **Zaključak.** Prikazana studija potvrdila je pozitivan efekat primene intenzivirane terapije insulinom na kontrolu metabolizma izraženu preko nivoa glikemije, glikozovanog hemoglobina (HbA1c) i fruktozamina, kao i preko antioksidativne/prooksidativne homeostaze. Ovo je dokaz da se adekvatnim izborom metode lečenja mogu prevenirati mnoge komplikacije dijabetesa izazvane slobodnim radikalima.

Ključne reči:
dijabetes melitus, tip 1; hipoglikemici; insulin; slobodni radikali; lipidi; peroksidacija; plazma; eritrociti; stres, oksidativni.

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Introduction

Many experimental and prospective clinical studies have proved so far that the increased production and sharp reduction of antioxidative defence mechanisms could play a critical role as a possible etiopathogenetic mechanisms for the development of insulin-dependent diabetes mellitus (IDDM) and its complications. Insulin-dependent diabetes mellitus as a chronic autoimmune disease is characterised by a chronic lymphocytic infiltration and consequent dysfunction and destruction of insulin-producing β-cells [1–4]. The histopathology of the islets at the onset of IDDM supports the view that soluble mediators of immunity that are liberated during the inflammatory lesion are selectively cytotoxic for β-cells. Among the cytokines the most cytotoxic is interleukin-1 beta (IL-lβ), which in synergy with tumor necrosis factor-alfa (TNF-α) or interferon gamma (IFN-γ) can generate free oxygen radicals. The generation of free oxygen radicals, together with the nitro oxide (NO) production are main secondary messengers of β-cells destruction [2, 5]. Pancreatic β-cells are especially vulnerable to free radicals because of their poor antioxidative capacity [6–8]. The susceptibility to vascular complications in diabetic patients has been shown to be dependent on antioxidant state [9, 10].

A design of an appropriate insulin replacement in insulin-dependent diabetes is successful if it mimics physiological insulin release. Physiologically, insulin is released in a pulsatile fashion. Conventional insulin treatment with fixed insulin dosage used to reduce sharply the mortality of diabetic coma, but not the occurrence of late diabetic complications, especially microangiopathy. Further improvement of metabolic fate includes functional insulin substitution. The results of prospective Diabetes Control and Complications Trial (DCCT) study, that has collected data from more than seven hundred patients, indicate that the program of intensive insulin therapy leads to better blood glucose control, stable metabolic control and near-normal hormonal responsivity [11]. The intensive insulin therapy, as a multiple daily insulin doses, was developed over the past years as suitable, acceptable and convenient [12, 18].

The study was undertaken to asses the effect of intensified insulin therapy regimen on pro-oxidant and antioxidant state in IDDM patients.

Methods

The present study included a total of 42 patients considered as insulin-dependent diabetics. Among them 24 were on intensive insulin therapy and the duration of diabetes was 7.85 ± 3.86 yr (range, 2–14 yr). They were put on multiple [4] daily insulin injection model by using short-acting insulin (Humulin R-Lilly) at each meal and intermediate-acting insulin (HumulinNPH-Lilly) at bed time. Mean insulin dose was 0.64 ± 0.07 IU/kgBW/24 h. Regular insulin was injected into the abdomen, while intermediate acting insulin was injected in the internal side of the thigh. Mean HbA1c was $7.5 \pm 1.03\%$. The patients on conventional insulin therapy [4] had the mean duration of diabetes of 5.73 ± 2.99 yr (range, 2–11yr). They received insulin two times per day (HumulinNPH-Lilly), in the morning and at bed time at the mean daily dose of 0.67 ± 0.09 IU/kgBW/24 h. Mean HbA1c was $8.8 \pm 2.08\%$.

All the examined patients were normotensive, with no evidence of coronary and peripheral arterial diseases. At the time of the study all the selected IDDM patients were negative at the clinical findings of microangiopathy, since after fundoscopy there was not a clinical findings of retinopathy. All the patients had also normal renal function (urine albumin was negative and plasma creatinine had the normal values). The control subjects (n =20) were healthy age-matched blood-donor volunteers.

All chemicals purchased for the study were of analytical grade purity, including 5.5 dithiobis nitrobensoic acid (DTNB), thiobarbituric acid (TBA), nitroblue tetrazolium (NBT), hydrogen peroxide and xanthine. They were purchased from Sigma-USA.

Blood samples: Heparinized venous blood was obtained by peripheral venipuncture and blood samples were always collected at 8.00 a.m, after 12 h of fasting. The obtained samples were immediately centrifuged at 2 000 g for 15 min, plasma was carefully removed and prepared erythrocytes were washed three times in physiological saline. The received plasma and erythrocytes were immediately processed for the analyses.

Estimation of reduced glutathione level in erythroctes (GSH RBC): The GSH RBC level was measured by spectrophotometric assay using DTNB. The packed erythrocytes (0.2 ml) were treated with the same volume of ice-cold H_2O and 1.3 ml of 5% TCA-1 mM EDTA. The samples were centrifuged and obtained supernatant was analysed for sulphydryl group content at 412 nm with DTNB dissolved in 0.1 M phosphate buffer. The level GSH RBC was expressed as μmol/gHb [5, 6].

Estimation of plasma antioxidative capacity (AOC): Plasma free radical-scavenging capacity-antioxidative capacity was tested by the modified procedure of Asakawa and Matsushita. The incubation study was carried at prooxidant conditions to determine the capacity of plasma to scavenge additional peroxides generated in liposome suspension after exposing to prooxidant conditions. For this purpose 1.5 ml of liposome suspension (from egg yolk at the concentration of 0.5 mg of total egg yolk phospholipids/ml), 0.2 ml of 0.5 M phosphate buffer pH 7.4 and 0.3 ml of the tested plasma sample were incubated at 37 °C for 30 min by adding 0.2 ml of 1 mM/l $FeCl_2$. Then, 1 ml of 30% TCA was added to the reaction mixture followed by centrifugation for 15 min at 10 000 g. One milliliter of 0.67% TBA was added to 1 ml of the received supernatant and the mixture was boiled at 97 °C for 20 min. Optical absorbance was measured at 532 nm. The antioxidative capacity was expressed as the percent of inhibition of lipid peroxidation, i. e. inhibition of malondialdehyade (MDA) formation compared with the control samples which contained phosphate buffer insted of plasma [7].

Estimation of plasma MDA products. Plasma lipid peroxidation was determined by thiobarbituric acid reactivity. Malondyaldehyde as an end product of fatty acid peroxidation can react with TBA to form a colored complex with a

maximum absorbance at 532 nm. The level of MDA was expressed as μmol/l [8].

Estimation of erythrocyte MDA products. Lipid peroxide products in erythrocytes were estimated by measuring TBA reactivity. For this purpose 0.4 ml of packed erythrocytes were suspended in 1.6 ml phosphate buffer saline pH 7.4 (to obtain 20% suspension of red blood cells). Then, 0.5 ml of 30% TCA was added, centrifuged and 2 ml of the obtained supernatant was transferred into another tube and 0.15 ml of 0.1 M/l edetic acid (EDTA) and 0.5 ml of 1% TBA (dissolved in 0.05 M NaOH) was added. The tubes were mixed and kept in a boiling water for 15 min. After the tubes were cooled the absorbance was read at 532 nm and 600 nm. The absorbance at 600 nm was substracted from the absorbance at 532 nm. Values of products MDA in nmol/ml RBC were determined using the extinction coefficient of MDA/TBA complex at 532 nm (91.56×10^5 per cm per molar solution). The results were expressed as nmol MDA/ml RBC [4].

Estimation of erythrocyte oxidative stress: For the estimation of erythrocyte susceptibility to H_2O_2-induced oxida-

Xanthine oxidase activity. It was measured according to the method of Kizaki and Sakurada [20], based on uric acid liberation when xanthine was used as substrate. The activity was expressed as U/l.

Statistical analysis. The results were expressed as mean $\pm$SD. The comparisons between the data of the examined groups were made using Student's t test and one-way ANOVA analysis of variance. The correlations were determined by linear regression analysis. A statistical significance was taken at $p < 0.05$.

Results

The group of IDDM patients having conventional insulin treatment is characterized with a more significant imbalance of plasma and erythrocyte antioxidant-prooxidant ratio than the group of IDDM patients having intensive insulin treatment, when compared to the control values (Table 1, Figures 1–2).

Table 1

Metabolic control (glycaemia, glycated hemoglobin-HbA1c and fructosamine), and antioxidative defence system (catalase activity in erythrocytes-catalase RBC, reduced gluthotione in erythrocytes-GSH RCB) parameters in investigated groups

Investigated parameters	Convetional (N=18)	Intensive (N=24)	Control subjects (N=20)
Glucose (mmol/l)	8.98±2.34 *** •	7.22±1.23	4.12±1.07
HbA$_{1c}$ (%)	8.8±2.08 •	7.5±1.03	
Fructosamine (U/gprot)	0.87±0.004 *** •	0.53±0.004 *	0.48±0.02
Catalase RBC (U/gHb)	11.05±0.92 ***	11.53±1.21 ***	13.40±2.21
GSH RBC (mmol/1Hb)	1.15±0.27 *	1.28±0.36 *	1.56±0.27
Plasma Xanthine oxidase (U/l)	3.02±0.57 *	2.67±0.47 *	2.02±0.24

$*p < 0.05$; $***p < 0.001$ – statistical significance compared to controls; • $p < 0.05$ – statistical significance compared to intensified insulin regimen

tive stress, erythrocytes were washed with isotonic saline solution containing 2.0 mM sodium azide to inhibit catalase activity. Washed erythrocytes were resuspended in saline-azide potassium phosphate buffer pH 7.4. One mililiter of 20% cell suspension was then preincubated for 10 min at 37°C. Peroxidative chalenge was induced by the addition of an equal volume (1.0 ml) of H_2O_2 in isotonic saline-azide solution (final H_2O_2 concentration 3.0 mM). The reaction was terminated by the addition of 0.5 ml of 30% TCA and the mixture was centrifuged. Values at MDA was detected in the obtained supernatant as a basic TBA reacting agent and it was used as an indicator of lipid peroxidation. The obtained results were expressed as nmol MDA/ml RBC [4].

Plasma and RBC catalase activity. It was estimated by the method of Goth [19], using hydrogen peroxide as substrate. The activity was expressed as U/l and U/gHb, respectively.
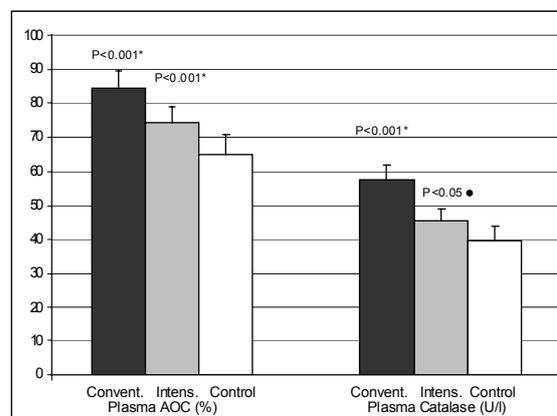

Fig. 1 – Plasma total antioxidative capacity (AOC) and plasma catalase activity
* statistical significance compared to controls; • statistical significance compared to intensified insulin regimen
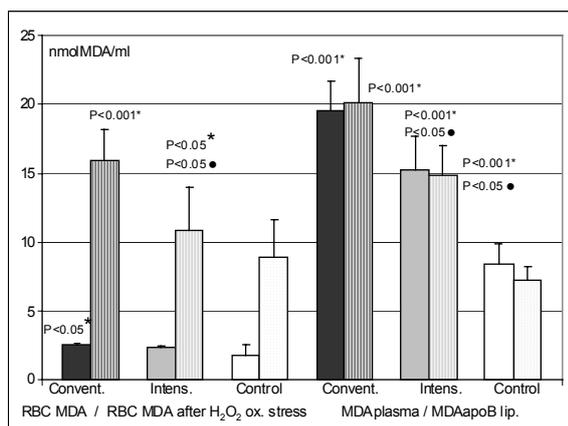
Fig. 2 – Malondialdehyde level in erythrocytes (MDA/RBC) at basal state and after H_2O_2-inuced oxidative stress; and malondialdehyde level in plasma (plasma MDA) and apoB-containing lipoproteins (apo B-lip MDA)

* statistical significance compared to controls; • statistical significance compared to intensified insulin regimen

A significant decrease in GSH RBC content and catalase activity was found in IDDM patients having conventional insulin treatment compared to controls, more than in the patients on intensive insulin regimen (Table 1). The obtained results are in agreement with the increased xanthine oxidase activity and the increased fructosamine level in the patients having conventional insulin regimen. The increase of plasma catalase activity can be explained by the lack of damaged erythrocytes, since in RBC of diabetic patients the catalase activity was lower, especially in the diabetic group on conventional treatment. The decresed level of total plasma antioxidative capacity in the group having conventional insulin treatment points out the deffect of the function to suppress oxidative stress and to scavenge generated free oxygen radicals (Figure 1). Both of IDDM groups had the increased plasma MDA level compared with the control value, but MDA concentration was higher in the patients having conventional insulin treatment more than in the patients having intensive insulin treatment (Figure 2). These results indicate an enhanced free radical production. The level of MDA in erythrocytes was examined by measuring TBA reactivity before and after H_2O_2-induced peroxidative challenge. A statistically significant increase in the concentration of MDA was found in both of groups of IDDM patients compared with the control value, but there was no significant difference between them. It is very important that after H_2O_2-induced oxidative stress, the cells of IDDM patients having conventional insulin treatment were the most susceptible ones to the induced oxidative stress, pointing to low efficacy in counteracting oxidative challenge.

Discussion

Glycated haemoglobin (HbA_{1c}), a marker of an average glycaemia, is a predictor of the late complications in diabetic individuals. The central role of HbA_{1c} in monitoring glycaemic control was obtained by the result of DCCT which followed 1441 insulin dependent diabetics between 1983 and 1993. It was found that an intensive metabolic control, compared with the standard control reduced the risk of development and progression of retinopathy, nephropathy and neuropathy by approximately 60%. It was also pointed out that the risk of complications rose with increasing HbA_{1c} [11]. Unlike glucose and urine ketone testing, glycated protein testing provides a quantitative measure of the average glycemia over and extended period of time (weeks or months), while unlike fructosamine, HbA_{1c} is unaffected by proteinuria. The elevated values obtained in the group on conventional insulin treatment indicate the risk for chronic complications associated with diabetes and the need for further improvement of the therapy (Table 1). Other studies confirmed these results [12, 13]. This is an important finding because the evaluation of metabolic control improves the targeting of preventive treatment.

Total plasma antioxidative capacity of the patients having intensive therapy was higher when compared with the capacity of the patients having conventional therapy, but yet lower than in the control subjects (Figure 1). Total plasma antioxidative capacity was expressed as the percent of the inhibition of MDA production in the suspension of phospholipids, which was initiated by the prooxidant conditions. The total antioxidative capacity of plasma (antioxidative defence mechanism) consists of enzymatic and nonenzymatic scavenger compounds. Therefore, the individual factors of enzymatic or nonenzymatic nature were not determined. The results of studies which correspond to ours reporting lower antioxidative capacity in the conditions considered unstable metabolic control (conventional insulin therapy) have demonstrated a decrease in the concentration of vitamin E, reduced glutathione, as well as superoxide dismutase (SOD) activity in IDDM patients [21–23]. It is documented that antioxidative enzymes of both, plasma and cells, suffer inactivation by the process of glycosylation. Some recent studies have also shown that higher glucose concentration decreases the radical scavenging activity of endothelial cells [17, 24].

A decreased concentration of plasma lipid peroxides in the patients having intensive insulin treatment surely contributes to the lower production of free radicals, due to better metabolic control. The intensive lipid peroxidation in diabetic patients on conventional therapy is most probably a consequence of the present hyperglycaemia and the consequential process of autooxidative glycosylation and depletion of nicotinamide adenine dinucleotide phosphate (NADPH) cell stores [22, 23, 25]. Free oxygen radicals are shown to reduce NO availability – one of the intracellular mechanisms of insulin action. In the presence of an increased amount of free radicals, the efficacy of insulin is decreased, since insulin is effective through NO production. In this way, the normalization of insulin sensitivity that is documented to occur during an intensive insulin treatment could be also explained. The decreased insulin requirements that have been also documented during intensive insulin therapy suggest that factors other than insulin deficiency contribute to normalization of insulin sensitivity. Among them is the decrease of free radical production [26–30]. On the other hand, the decrease in the level of NO can lead to vasoconstriction and platelet aggregation – the concept of "peroxide vascular tone". Hypercho-

lesterolemia – particularly hyperbetalipoproteinemia – is an important causative factor in atherogenesis and leading risk of coronary heart disease. The oxidative modification hypothesis originally based on the finding that cell-modified (oxidized) LDL had the potential to cause foam cell formation [31, 32]. In our study an increased MDA level was present not only in plasma and RBC but especially in apo-B containing lipoproteins, as it was demonstrated by their precipitation and MDA level quantification.

The results obtained in this study point out a significantly higher GSH level in erythrocytes of diabetic patients having intensive insulin treatment compared to those on conventional treatment (Table 1). Erythrocytes contain a high concentration of glutathione in its reduced form. Reduced glutathion is involved in the glutathione redox cycle which plays a role in protecting Hb from erythrocytes against oxidation [22, 30]. An oxidized form of GSH (GS-SG) can be converted to GSH by the enzyme glutathione reductase in a NADPH-dependent reaction. Aldose reductase, the first enzyme in the polyol pathway also requires NADPH as a cofactor, catalyzing the reduction of glucose to sorbitol. This pathway is involved in the pathogenesis of diabetic complications [1]. During hyperglycaemia there is increased flux through the polyol pathway and accumulation of sorbitol. An increased polyol pathway activity results in the depletion of NADPH with the consequent mayor alteration of erythrocyte redox-potential, by the inhibition of glutathione reductase activity. This leads to an intracellular deficiency of GSH due to an insufficient GSH regeneration. Another cause of the decreased level of GSH could be a reduced synthesis. A reduced NADPH synthesis as a consequence of the decreased activity of glucose-6-phosphate dehydrogenase was also documented in diabetes mellitus. It is possible that the intensive insulin therapy, besides good metabolic control, contributes to an adequate regeneration of GSH from GS-SG during the process of its reduction by GSH reductase and NADPH. The importance of an adequate GSH level comes from the conclusion that GSH exerts insulinotropic effect, since it is capable of potentiating glucose-induced insulin secretion in a dose-related manner, both *in vivo* and *in vitro*.

This effect is related to Ca^{++} uptake via the voltage-dependent Ca^{++} - channels [24, 30].

An increased xanthine oxidase activity was documented in both groups, but in the group on conventional insulin regimen the increase was more pronounced. Xanthine oxidase is a limiting enzyme in a terminal step of purine metabolism. Beside the production of uric acid, this enzyme is responsible for generation of superoxide. The importance of xanthine oxidase as a very powerful generator of oxidative stress is documented by the results of protective effects of allopurinol, a competitive inhibitor of xanthine oxidase in type-1 diabetes [33].

A number of studies point out an increased blood viscosity, reduced erythrocyte life span and increased adhesion, what can have a role in the rheological impairment and ischemia of certain tissues in diabetic patients [34]. Previous studies reported a significant correlation between the glucose-induced membrane lipid peroxidation and the increased osmotic fragility of erythrocytes. The exposure of erythrocytes to oxidative stress allows a better evaluation of the total antioxidative system of erythrocytes [35–37]. The increased level of erythrocyte lipid peroxidation as a response to H_2O_2-induced stress in the patients may verify that erythrocytes of the patients on an intensive insulin treatment are more able to counteract oxidative stress than the erythrocytes of patients having conventional therapy type. On the other hand, oxidatively damaged red cells bound avidly to macrophage receptors. Several other lines of evidence supported the interpretation that and oxidatively damaged red cells were bound to the same receptor or receptors as oxidatively modified LDL [31].

Coclusion

In conclusion, the presented data suggest that rational therapeutic applications may prevent oxidative stress and increased generation of free radicals. It may contribute to the delay in the development of micro- and macroangiopathy and their consequences in insulin-dependent diabetics, increased quality of life and decreased additional therapy expenditures.

R E F E R E N C E S

1. *Baynes JW*. Role of oxidative stress in development of complications in diabetes. Diabetes 1991; 40(4): 405–12.
2. *Rossini AA, Mordes JP, Handler ES*. Speculations on etiology of diabetes mellitus. Tumbler hypothesis. Diabetes 1988; 37(3): 257–61.
3. *Haskins K, Bradley B, Powers K, Fadok V, Flores S, Ling X*, et al. Oxidative stress in type 1 diabetes. Ann N Y Acad Sci 2003; 1005: 43–54.
4. *Gil-del Valle L, de la C Milian L, Toledo A, Vilaro N, Tapanes R, Otero MA*. Altered redox status in patients with diabetes mellitus type I. Pharmacol Res 2005; 51(4): 375–80.
5. *Wolff SP, Dean RT*. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. Biochem J 1987; 245(1): 243–50.
6. *Erciyas F, Taneli F, Arslan B, Uslu Y*. Glycemic control, oxidative stress, and lipid profile in children with type 1 diabetes mellitus. Arch Med Res 2004; 35(2): 134–40.
7. *Giugliano D, Ceriello A, Paolisso G*. Oxidative stress and diabetic vascular complications. Diabetes Care 1996; 19(3): 257–67.
8. *Hunt JV, Dean RT, Wolff SP*. Hydroxyl radical production and autoxidative glycosylation. Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. Biochem J 1988; 256(1): 205–12.
9. *Rasheed Z, Ali R*. Reactive oxygen species damaged human serum albumin in patients with type 1 diabetes mellitus: biochemical and immunological studies. Life Sci 2006; 79(24): 2320–8.
10. *Pavlović D, Kocić R, Kocić G, Jevtović T, Radenković S, Mikić D*, et al. Effect of four-week metformin treatment on plasma and erythrocyte antioxidative defense enzymes in newly diagnosed obese patients with type 2 diabetes. Diabetes Obes Metab 2000; 2(4): 251–6.
11. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-

dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993; 329(14): 977–86.

12. *Kulenović I, Rašić S, Grujić M*. Metabolic control and body mass index in patients with type 1 diabetes on different insulin regimens. Bosn J Basic Med Sci 2004; 4(1): 23–8.

13. *Schiel R, Franke S, Appel T, Voigt U, Ross IS, Kientsch-Engel R*, et al. Improvement of the quality of diabetes control and decrease in the concentrations of AGE-products in patients with type 1 and insulin-treated type 2 diabetes mellitus: results from a 10 year-prospective, population-based survey on the quality of diabetes care in Germany (JEVIN). Eur J Med Res 2004; 9(8): 391–9.

14. *Andreeva LI, Kozhemiakin LA, Kishkun AA*. Modification of the method of determining lipid peroxidation in a test using thiobarbituric acid. Lab Delo 1988; (11): 41–3. (Russian)

15. *Jackson RC*. Studies in the enzymology of glutathione metabolism in human erythrocytes. Biochem J 1969; 111(3): 309–15.

16. *Beutler E, Duron O, Kelly BM*. Improved method for the determination of blood glutathione. J Lab Clin Med 1963; 61: 882–8.

17. *Yoshida K, Hirokawa J, Tagami S, Kawakami Y, Urata Y, Kondo T*. Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux. Diabetologia 1995; 38(2): 201–10.

18. *Asakawa T, Matsushita S*. Thiobarbituric acid test for detecting lipid peroxides. Lipids 1980; 14(4): 401–6.

19. *Goth L*. A simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta 1991; 196(2–3): 143–51.

20. *Kizaki H, Sakurada T*. Simple micro-assay methods for enzymes of purine metabolism. J Lab Clin Med 1977; 89(5): 1135–44.

21. *Costagliola C, Libondi T, Menzione M, Rinaldi E, Auricchio G*. Vitamin E and red blood cell glutathione. Metabolism 1985; 34(8): 712–4.

22. *Costagliola C*. Oxidative state of glutathione in red blood cells and plasma of diabetic patients: in vivo and in vitro study. Clin Physiol Biochem 1990; 8(4): 204–10.

23. *Hiramatsu K, Arimori S*. Increased superoxide production by mononuclear cells of patients with hypertriglyceridemia and diabetes. Diabetes 1988; 37(6): 832–7.

24. *Ammon HP, Klumpp S, Fuss A, Verspohl EJ, Jaeschke H, Wendel A*, et al. A possible role of plasma glutathione in glucose-mediated insulin secretion: in vitro and in vivo studies in rats. Diabetologia 1989; 32(11): 797–800.

25. *Jain SK, Levine SN, Duett J, Hollier B*. Elevated lipid peroxidation levels in red blood cells of streptozotocin-treated diabetic rats. Metabolism 1990; 39(9): 971–5.

26. *Paolisso G, Giugliano D*. Oxidative stress and insulin action: is there a relationship? Diabetologia 1996; 39(3): 357–63.

27. *Ammon HP, Hagele R, Youssif N, Eujen R, El-Amri N*. A possible role of intracellular and membrane thiols of rat pancreatic islets in calcium uptake and insulin release. Endocrinology 1983; 112(2): 720–6.

28. *Ammon HP, Hehl KH, Enz G, Setiadi-Ranti A, Verspohl EJ*. Cysteine analogues potentiate glucose-induced insulin release in vitro. Diabetes 1986; 35(12): 1390–6.

29. *Ookawara T, Kawamura N, Kitagawa Y, Taniguchi N*. Site-specific and random fragmentation of Cu,Zn-superoxide dismutase by glycation reaction. Implication of reactive oxygen species. J Biol Chem 1992; 267(26): 18505–10.

30. *Murakami K, Kondo T, Ohtsuka Y, Fujiwara Y, Shimada M, Kawakami Y*. Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus. Metabolism 1989; 38(8): 753–8.

31. *Merzouk S, Hichami A, Sari A, Madani S, Merzouk H, Yahia Berrouiguet A*, et al. Impaired oxidant/antioxidant status and LDL-fatty acid composition are associated with increased susceptibility to peroxidation of LDL in diabetic patients. Gen Physiol Biophys 2004; 23(4): 387–99.

32. *Steinberg D. Lewis A*. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. Circulation 1997; 95(4): 1062–71.

33. *Desco MC, Asensi M, Marquez R, Martinez-Valls J, Vento M, Pallardo FV*, et al. Xanthine oxidase is involved in free radical production in type 1 diabetes: protection by allopurinol. Diabetes 2002; 51(4): 1118–24.

34. *Jones RL, Peterson CM*. Hematologic alterations in diabetes mellitus. Am J Med 1981; 70(2): 339–52.

35. *Wautier JL, Paton RC, Wautier MP, Pintigny D, Abadie E, Passa P*, et al. Increased adhesion of erythrocytes to endothelial cells in diabetes mellitus and its relation to vascular complications. N Engl J Med 1981; 305(5): 237–42.

36. *Satoh M, Imaizumi K, Bessho T, Shiga T*. Increased erythrocyte aggregation in diabetes mellitus and its relationship to glycosylated haemoglobin and retinopathy. Diabetologia 1984; 27(5): 517–21.

37. *Wali RK, Jaffe S, Kumar D, Kalra VK*. Alterations in organization of phospholipids in erythrocytes as factor in adherence to endothelial cells in diabetes mellitus. Diabetes 1988; 37(1): 104–11.