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Biomedicine & Pharmacotherapy 58 (2004) 539–545
BIOMEDICINE & PHARMACOTHERAPY
http://france.elsevier.com/direct/BIOPHA/

Dossier: Diabetes: Basic Research and Clinical Approach

Aspects of oxidative stress in children with Type 1 diabetes mellitus

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Received 16 September 2004

Available online 21 October 2004

Abstract

Diabetes mellitus is considered to be one of a rank of free radical diseases. The existence of hyperglycemia produces increased oxidative stress (OS) via non-enzymatic glycation, glucose autoxidation, and alterations in polyol pathway activity with subsequent influences on the whole organism. In childhood, Type 1 diabetes prevails and is characterized by its autoimmune character with progressive destruction of β cells and lack of insulin in genetically predisposed patients. Our study focused on diabetic children and their 1st degree relatives and confirmed increased oxidative stress in diabetic children as well as a similar tendency in their siblings. Following this, we carried out a one-year study comprising diabetic children supplemented with vitamins E and C. The vitamin treatment improved diabetes control and reduced markers of oxidative stress substantially when compared with non-supplemented diabetic children. As oxidative stress impairs not only lipids and proteins, but also DNA, we attempted to examine the level of DNA strand breaks as well as DNA repair processes using comet assay modifications. Though children with Type 1 diabetes demonstrated increased oxidative stress (lower SOD and GSH when compared with healthy children), their oxidative DNA damage (measured as DNA strand breaks) were not substantially altered compared with normals. On the other hand, their DNA repair capacity was significantly increased. This demonstrates a stimulated DNA repair process that is most certainly a response to the permanently elevated state of oxidative stress. Owing to the presented results, it is appropriate to ponder the increased influence of oxidative stress on children with Type 1 diabetes and to take into account this fact when considering their treatment.

Keywords: Type 1 diabetes mellitus; Oxidative stress; DNA repair

1. Introduction

Diabetes mellitus is a metabolic disease characterized by hyperglycemia. This major finding results not only in typical clinical symptoms, but also in major biochemical changes including prolonged OS via non-enzymatic glycation and AGEs production, glucose and AGEs autoxidation, and alterations in polyol pathway activity with a subsequent influence on the organism as a whole [15,17,20,22–24].

In childhood, Type 1 diabetes (T1DM) prevails and is characterized by its autoimmune character with progressive destruction of β cells and lack of insulin in genetically predisposed patients [3]. Prolonged OS due to the above-mentioned consequences of hyperglycemia and autoimmune inflammation contributes to biomolecular injury (proteinic, lipidic and DNA damage with further pathogenetic processes such as cell apoptosis, changes in cell metabolic activity and cell signaling, aging,...) [14,31,32]. The defense of the organism against OS is ensured by means of a large scale of antioxidant substances and enzymes rectifying previously arisen damage [31]. Reduced plasma antioxidant capacity present in diabetic patients has been repeatedly confirmed. This is due to an increased consumption of distinct antioxidant components (e.g. intracellular glutathione) or to primarily low levels of antioxidant substances (flavonoids, carotenoids, vitamin E and C) [1]. To ensure a substantial

Abbreviations: OS, oxidative stress; T1DM, Type 1 diabetes mellitus; SOD, superoxide dismutase; GSHPx, glutathione peroxidase; AOC, plasma antioxidant capacity; GSH, reduced glutathione; MDA, malondialdehyde; DNAsb, DNA strand breaks; DNArc, DNA repair capacity; DNRI, DNA repair index.

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doi:10.1016/j.biopha.2004.09.011

antioxidant effect, it is necessary to supply two distinct factors simultaneously acting in reciprocal oxidation and reduction and assuring in that way their sufficient regeneration [26]. The repair of DNA oxidative damage is sustained by an extensive array of repair enzymes [35]. Presently, it is possible to use various modifications of the comet assay method for the evaluation of oxidative DNA damage and DNA repair capacity of the organism [7].

Our workplace pursued an intensive research in the field of oxidative stress in patients with a range of diseases, and especially in diabetes mellitus as a model of a free radical disease. The aim of our research was (1) to study parameters of oxidative stress in Type 1 diabetic children and their 1st degree relatives, (2) to find out whether one-year supplementation with vitamins E and C would improve OS status and diabetes control, (3) to measure oxidative DNA damage and the intensity of DNA repair process in diabetic children.

2. Methods

The following laboratory methods were used in the course of our studies in diabetic patients:

(1) Markers of diabetes control: *glycated hemoglobin* (HbA_{1c}) was determined using the HPLC method (Tosoh corp.; Tokyo, Japan) and *glycated protein* (fructosamine) by photometric analysis (Roche; Manheim, Germany). The upper limits showing good diabetes compensation were 6.5% for HbA_{1c} and 320 μmol/l for glycated protein. *Glycaemic index* (GCI) was calculated as GCI = M + (Glu_{max} − Glu_{min})/2, M = arithmetic mean out of seven glycaemias, Glu_{max} = maximum daily glycaemia, Glu_{min} = minimum daily glycaemia, values GCI up to 10.0 give evidence of good diabetic control, GCI higher than 20.0 shows very bad control). For *basic biochemical parameters*, common routine photometric methods were used.

(2) *Oxidative stress markers*: superoxide dismutase (SOD), glutathione peroxidase (GSHPx), plasma antioxidant capacity (AOC), reduced glutathione (GSH), and malondialdehyde (MDA) were assessed. Kits from Randox (Crumlin, Great Britain), adapted to an automated Hitachi 717 analyzer (Roche, Mannheim, Germany) were used for determination of SOD in erythrocytes, GSHPx in whole blood and AOC. GSH concentration in erythrocytes was estimated using kits from Oxis Int. (Bonnenie, Marue, France), while plasma MDA, as a product of lipid peroxidation, was determined photometrically as thiobarbituric acid reactive substances (TBARS) [34].

(3) *Vitamin E* determination in serum by reversed-phase HPLC was used (column Supelco (Bellefonte, PA, USA) LC-ABZ 250 × 4.6 mm; 5 μm particle size) with UV detection at 292 nm. The extraction was carried out (after addition of internal standard) with hexane. The analyses were performed on a component system consisting of a Module SP 8770 pump, UV 2000 detector, SP 8875 autosampler and SP 4100 integrator (Spectra Physics, San Jose, CA, USA). Vitamin C was impossible to determine due to technical problems.

(4) *DNA strand breaks* (DNAsb) measured by comet assay (alkaline single-cell gel electrophoresis) [7,8,11] in peripheral blood lymphocytes isolated from heparinized blood samples were used as a parameter of oxidative DNA damage. Results are expressed as strand breaks per 10^9 Da of single stranded DNA [6]. Cells embedded in a thin layer of agarose on a microscope slide were lysed with detergent-containing hypertonic salt solution, DNA was unwinded under alkaline conditions and the resulting nucleoids electrophoresed at high pH (see [6,7] for details]. DNA was pulled out of the nucleoid by electrophoresis to form a "comet tail". Comets were visualized using fluorescence microscopy, after staining with ethidiumbromide (Sigma). The relative amount of DNA in the tail compared to the head demonstrated the number of DNA breaks present.

The individual *DNA repair capacity* (DNArc) restoring oxidative DNA damage was measured using a modified comet assay using cell extracts from patients' lymphocytes. In this case, cultured HeLa cells treated with a photosensitiser Ro 19-8022 Hoffman-La Roche and visible light were used as target cells with a defined amount of oxidative damage. These cells embedded in agarose were treated for 45 min with cell extracts prepared from lymphocytes isolated from tested patients. The number of DNA breaks (expressed as strand breaks per 10^9 Da) unmasked in the DNA of target cells by cell extracts expressed the capacity of the examined lymphocytes to initiate the repair of oxidized bases (8-oxoguanines) [10,11].

To express the relationship between the intensity of individual DNA repair capacity and the existing level of oxidative DNA damage, a *DNA repair index* (DNRI) was formulated as DNRI = value of DNArc/value of DNAsb.

Blood samples were taken in all diabetic participants on the same day from 7.30 a.m. to 9.30 a.m. and immediately transported to the laboratories. OS markers, vitamin E level, and biochemical parameters were performed at the Institute of Clinical Biochemistry and Haematology of the Charles University Hospital in Plzeň. Evaluation of DNAsb and DNArc was performed in the Department of Toxicology at the Purkynje Military Academy in Hradec Králové.

OS markers, vitamin E, DNAsb as well as DNArc in diabetic patients were compared with those in healthy controls.

2.1. Statistics

Data were expressed as mean ± standard deviation. Wilcoxon unpaired test, Spearman and Kruskal–Wallis test and rank correlation were used in statistical evaluation of the results. Statistical significance was implied by P-value <0.05.

2.2. Ethics Committee

The Local Ethics Committee approved all three studies, and patients' informed consent was obtained from all participants.

2.3. Study population

2.3.1. Study of parameters of oxidative stress in diabetic children and their 1st degree relatives

This study was published in January 2003 in the *Journal of Diabetes and its Complications* [34]. As this material is related to further data, we briefly summarize the population and results.

A group of 50 children with Type 1 DM (21 girls and 29 boys, aged 2.5–19.5 years, mean age 11.96 ± SD 4.69 years) from the West Bohemian Region were examined for oxidative stress parameters (SOD, GSHPx, AOC, GSH, MDA). The mean time of disease duration was 51 months, one child was newly diagnosed with Type 1 DM. The longest duration of disease was 14 years.

Patients' parents without diabetes (65 persons—41 mothers and 24 fathers, mean age 38.74 ± SD 6.01 years) as well as their 32 siblings also without diabetes (20 girls and 12 boys, mean age 13.69 ± SD 4.41 years) were examined within the same period for oxidative stress parameters.

The results of oxidative stress parameters in diabetic children and their siblings were compared with an age- and sex-matched group of 30 healthy children whose parameters of oxidative stress were measured before planned hernia or phimosis surgery. Parameters of oxidative stress in parents were compared with 35 adult healthy volunteers and with their diabetic children.

2.3.2. Study of vitamin E and C one-year supplementation in diabetic children

Thirty-eight diabetic children (19 boys, 19 girls), mean age 12.5 ± SD 4.17 years with poor compensation of DM 1 (HbA_{1c} 11.55 ± SD 2.70%, glycated protein 382 ± SD 60.9 μmol/l), mean disease duration 2.4 years, were involved in the study. In the period from 2/2000 to 5/2001, the substitution study with vitamins E and C was carried out every 3 months (spring–summer), then 3 months break, then again substitution for 3 months (autumn–winter), then 3 months break. The dosage of vitamins depended on body weight: up to 25 kg body weight 1 capsule of vitamin E per 100 mg and 1 tablet vitamin C per 100 mg, up to 50 kg 2 × 1 both vitamins, over 50 kg body weight 3 × 1 both vitamins. Before and after each 3-month substitution, the assessment of markers of oxidative stress OS (SOD, GSH, GSHPx, AOC, MDA), levels of vitamin E, HbA_{1c}, glycated protein was completed.

Fourteen children with Type 1 DM (six girls and eight boys), mean age 13.07 ± SD 4.57 years, mean HbA_{1c} 9.64 ± SD 2.66%, glycated protein 295.6 ± 88.7 μmol/l, mean disease duration 2.2 years, served as a control group. They did not take vitamin E and C supplementation for different reasons. No child with new onset diabetes was enrolled in the study.

All children were on an intensified insulin regimen (4–5 dosages of rapid and medium-acting insulin per day) and diabetic regulated diet. There was no other special approach to children except for the first instruction before starting vitamin supplementation. Regular check-ups were as in non-supplemented children every 3 months.

2.3.3. Study of oxidative DNA damage and DNA repair capacity in diabetic children

Twenty diabetic children of Type 1 randomly chosen from diabetic patients treated at the Department of Pediatrics, Charles University Hospital in Pilsen (eight girls and 12 boys, aged 9.5–19 years, mean age 13.26 ± SD 2.98 years, mean HbA_{1c} 9.50 ± SD 2.52%) were examined for parameters of OS, oxidative DNA damage (DNAsb) and DNA repair capacity (DNArc) and calculated index DNRI. The results were compared with a group of age- and sex-matched 11 healthy children (five girls, six boys, aged 9.5–19 years, mean age 13.73 ± SD 3.80 years). Besides this basic comparison, the outcomes of diabetic children were matched with the results of the same parameters in adult Type 1 diabetic patients: group A (23 patients without diabetic microvascular complications, mean age 37.09 ± 10.92 years, duration of diabetes 14.70 ± 3.11 years, mean HbA_{1c} 8.11 ± 0.98%) and group B (30 patients with diabetic microvascular complications, mean age 41.07 ± 11.33 years, duration of diabetes 17.63 ± 2.57 years, mean HbA_{1c} 8.33 ± 1.09%). Both groups were comparable with regard to their diabetes control, age, sex and insulin dose, but significantly differed in disease duration ($P < 0.01$).

3. Results

3.1. Study of parameters of oxidative stress in diabetic children and their 1st degree relatives

The results in diabetic children showed significantly lower GSHPx, AOC and increased MDA compared with healthy children. Similar findings were found in their siblings though without statistical significance (Fig. 1). The parents of diabetic children had normal parameters of oxidative stress.

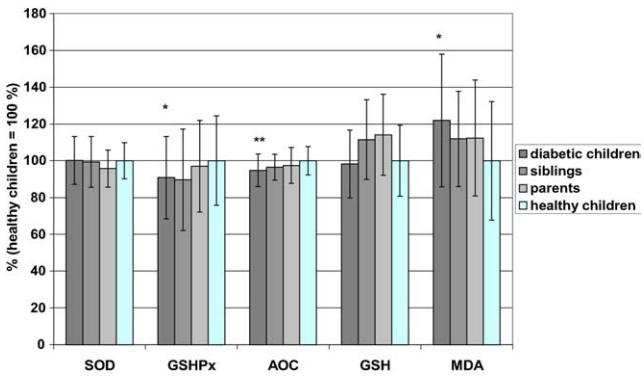

Fig. 1. Parameters of oxidative stress in T1DM children, their siblings and parents. Healthy children values = 100%. * $P < 0.05$, ** $P < 0.01$ T1DM children versus healthy children.

Table 1
Development of diabetes control and parameters of oxidative stress before and after supplementation with vitamins E and C

2000–2001	HbA_{1c} (%)	Glycated protein (μmol/l)	SOD (U/g Hb)	GSHPx (U/g Hb)	AOC (mmol/l)	GSH (mmol/l)	MDA (μmol/l)
Before spring–summer	11.55 ± 2.70	382 ± 60.1	1352 ± 150	58.61 ± 14.33	1.42 ± 0.14	1.59 ± 0.24	2.90 ± 0.96
After spring–summer	10.52 ± 1.98	357.5 ± 92.9	1302 ± 130	61.70 ± 13.40	1.43 ± 0.11	1.57 ± 0.18	2.77 ± 0.52
Before autumn–winter	10.23 ± 1.90	317.8 ± 72.8	1222 ± 157	59.72 ± 13.49	1.457 ± 0.09	1.76 ± 0.37	2.66 ± 0.44
After autumn–winter	10.18 ± 1.90 *	298.5 ± 60.2 **	1377 ± 177 *	58.18 ± 13.73	1.43 ± 0.07	1.81 ± 0.36 **	2.56 ± 0.61

* $P < 0.05$. ** $P < 0.01$.

3.2. Study of vitamin E and C one-year supplementation of diabetic children

Children supplemented during 1 year with vitamin E and C demonstrated improved diabetes control (HbA_{1c} $P < 0.05$ and glycated protein $P < 0.01$). Their parameters of oxidative stress improved significantly: SOD $P < 0.05$, GSH $P < 0.01$ (Table 1) after 1 year in the study. The level of vitamin E in serum was significantly higher at the end of each supplementation period (Table 2). In children without supplementation, HbA_{1c} and glycated protein were also measured every 3 months, but there were unsubstantial changes in these values. The only value which altered at the end of 1–years investigation was the glycated protein ($P < 0.05$) (Table 3).

3.3. Study of oxidative DNA damage and DNA repair capacity in diabetic children

Children with Type 1 diabetes had lower SOD ($P < 0.001$) and GSH ($P < 0.05$) than healthy controls. The other parameters of oxidative stress were without substantial differences (AOC, GSHPx, MDA). Oxidative DNA damage, expressed as DNA strand breaks and measured using the comet assay, were also unchanged in diabetic patients. On the other hand, DNA repair capacity examined using a modified comet assay was significantly increased in Type 1 diabetic children ($P < 0.05$). This finding expresses stimulated DNA repair process restoring DNA damage (Fig. 2). Finally, we tried to compare the intensity of oxidative stress and DNA tests in Type 1 DM children with the two groups of Type 1 diabetic adults: group A without diabetic microvascular complications and group B with diabetic complications (retinopathy, nephropathy, neuropathy or combinations of the three). It is obvious that though there was worse diabetes control in Type 1 diabetic children (HbA_{1c} 9.50 ± 2.52%) when compared with both adult groups, there was no difference in markers of oxidative stress in children and adults—except for the level of MDA (in children lower than in adults without complications $P < 0.05$). No difference in DNA breaks among all three groups was found, but the intensity of DNA repair process was substantially elevated in children versus group A ($P < 0.01$) and especially versus group B (adults with complications) ($P < 0.001$). DNRI balancing the intensity of repair process against DNA damage was higher in children versus adults without complications ($P < 0.01$) and especially very high in children versus adults with diabetic complications ($P < 0.001$) (Fig. 3).

Table 2
Values of vitamin E in diabetic children with the applied supplementation

2000–2001	Vitamin E (mg/l)
Before supplementation spring–summer	5.47 ± 1.66
After supplementation spring–summer	8.24 ± 2.96 ***
Before supplementation autumn–winter	5.57 ± 1.29
After supplementation autumn–winter	7.04 ± 2.45 **

**$P < 0.01$. ***$P < 0.001$

4. Discussion

4.1. Oxidative stress in Type 1 diabetic children

4.1.1. Study of oxidative stress parameters in diabetic children and their 1st degree relatives

As already published by our group [34], we confirmed evidence of impaired antioxidative protection in diabetic children in accordance with other authors [1,12] and similar tendency of low antioxidative defense in siblings of diabetic children (low GSHPx levels and AOC).

4.1.2. Study of vitamin E and C one-year supplementation in diabetic children

On the basis of our previous study and other papers [2,13,16], we suggested one-year supplementation study with vitamins E and C in our children with poorly controlled Type 1 diabetes. It is better to apply both vitamins simulta-

Table 3
Comparison of T1DM compensation in children with vitamin substitution and without substitution

2000–2001	Children without vitamins		Children with vitamins E, C	
	HbA_{1c} (%)	Glycated protein (μmol/l)	HbA_{1c} (%)	Glycated protein (μmol/l)
Before spring–summer	9.64 ± 2.65	295.6 ± 88.7	11.55 ± 2.70	382.0 ± 60.1
After spring–summer	9.36 ± 1.83	307.0 ± 69.9	10.52 ± 1.98	357.5 ± 92.9
Before autumn–winter	8.78 ± 1.58	277.0 ± 63.7	10.23 ±1.90	317.8 ± 72.8
After autumn–winter	9.15 ± 1.96	319.3 ± 56.7 *	10.18 ± 1.90 *	298.5 ± 60.2 **

* $P < 0.05$. ** $P < 0.01$.

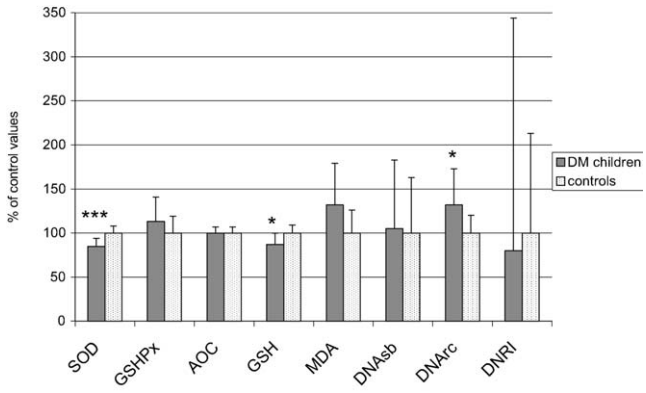

Fig. 2. Parameters of oxidative stress, DNA damage and DNA repair in T1DM and healthy children. * $P < 0.05$, ** $P < 0.001$ T1DM children versus healthy children (healthy children values = 100%).

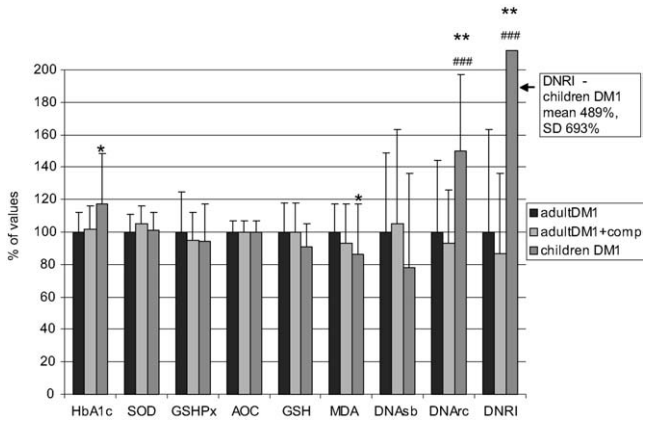

Fig. 3. Parameters of oxidative stress, DNA damage and DNA repair in T1DM adults without complications, with complications and T1DM children. T1DM adults without complication values = 100%. * $P < 0.05$, ** $P < 0.01$ T1DM children versus adult T1DM without diabetic microvascular complications. ### $P < 0.001$ T1DM children versus adult T1DM with complications.

neously because of their reciprocal reduction and oxidation in antioxidant network [25,26,28]. The study design was extended until 1 year and we proved significantly elevated levels of vitamin E in serum, improvement in OS markers (SOD, GSH) and better diabetes control (HbA_{1c} $P < 0.05$ and also glycated protein $P < 0.01$) at the end of the study considering also the fact that children with supplementation had otherwise the same therapeutic approach as the control group. Our findings are similar to those published as the results of shorter supplementation period [4,5,18,19,29]. On the other hand, the outcomes of supplementation studies in older populations are not always convincing [33]. The positive influence of vitamin E and C supplementation in the older age group may be influenced through chronic exposure to environmental toxins and other factors (smoking, air pollution, chronic disease, etc.).

4.1.3. Study of oxidative DNA damage and DNA repair capacity in diabetic children

Oxidative DNA damage has been studied for several years in diabetic population with important findings of increased DNA injury expressed as strand breaks DNAsb [6–9,21–23,27,36].

DNA repair is a complex process restoring DNA damage due to different toxic factors including oxidative stress. It has been proved that each human individual has his own particular DNA repair capacity [10]. DNA repair absence or its incapacity leads to impaired cell metabolism and to cell apoptosis or even to completely disorganized cell actions leading to carcinogenesis [30,31]. So far, DNA repair capacity has been studied in humans in relation to possible carcinogenesis or in certain genetic syndromes such as xeroderma pigmentosum, Nijmegen breakage syndrome or ataxia teleangiectatica where low DNArc is present and cells after irradiation are unable to restore arising DNA damage. Cell death or cell proliferation is then induced and leads to tissue defects or carcinogenesis [30].

As there are no studies about DNA repair capacity in diabetic individuals with regard to T1DM course and development of complications or even tendency to carcinogenesis, we examined parameters of OS, DNAsb and DNArc in a group of 53 Type 1 diabetic adults. Though being aware of a large scale of very specific DNA repair enzymes, we took advantage of a modified comet assay method expressing the capacity of the examined lymphocytes to initiate the repair of oxidized bases (8-oxoguanines measuring) [7,9,11]. The population of the study was divided into group A (23 patients without diabetic microvascular complications) and group B (30 patients with complications) and evaluated the intensity of OS, DNA damage and DNArc, and the acquired results were compared with the control group of age- and sex-matched healthy individuals and finally between themselves (Varvařovská J, et al., submitted for publication). DNA repair capacity was significantly higher in Type 1 diabetic subjects than in healthy controls. The DNRI reflecting the intensity of repair process to the extent of DNA damage was also increased in our patients especially in those without complications. These findings could represent the balance between the influence of OS on DNA damage and prompted repair capacity. The subgroup of patients with complications had lower DNArc but still higher than the control group. This finding indicates a less intensive repair process possibly due to its overloading, exhaustion or deficiency. The results in question (submitted for publication) were then compared with our diabetic children. It was obvious that though pediatric patients had worse diabetes control, they had higher SOD levels and lower MDA than diabetic adults, i.e. less oxidative stress. Their DNA damage was not substantially different from the results of the adults. Furthermore, DNA repair capacity in children was higher than in T1DM adults without complications ($P < 0.05$), and when compared with T1DM adults with microvascular complications the difference was still higher ($P < 0.001$). This finding indicates either better antioxidative defense including DNA repair process in young organisms, or shorter duration of disease and hence less exhausted repair enzymes due to permanent oxidative stress.

When the results of diabetic children were compared with healthy pediatric controls, significantly lower SOD and GSH

and stimulated DNArc were found in T1DM children as an apparent sign of important oxidative stress. Unfortunately, there are no consistent published data both in children and adults for comparison.

5. Conclusion

Considering the presented results, it is appropriate to ponder about the increased influence of oxidative stress on children with Type 1 diabetes and to take this detail into account when determining their treatment.

5.1. Conflict of interest statement

None declared.

Acknowledgments

This research was supported by Research Aim MSMT 111 40001 Study of Factors Influencing Metabolic Disturbances and Their Possible Modification, Charles University Prague, Faculty of Medicine Plzen, Czech Republic and by IGA MZ CR grant NR 7919-3/2004, Individual DNA repair capacity induced by oxidative stress and its influence on the development and prognosis of chronic non-infectious diseases.

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