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## Introduction

Free radicals and other reactive species are thought to play an important role in many human diseases. A serious imbalance between production of reactive species and the antioxidant protective system due to increased production of reactive species or low levels of antioxidants leads to oxidative damage (oxidative stress, OS) and development of various disorders (Halliwell, 2004). Therefore, the evaluation of OS could be used as a nonspecific marker of systemic disorders in the human body. Moreover, if the standard treatment shows itself as not sufficient to decrease an ongoing OS, the applied therapy may require modification, for example, by additional administration of vitamins, antioxidants, etc. We believe that some parameters of the blood antioxidant status could be used for evaluation of OS.

It is particularly important to estimate OS in GHD children. Treatment with recombinant growth hormone (rGH) and a subsequent increase in linear growth rate can lead to acceleration of metabolic processes and be accompanied by changes in the blood antioxidant status parameters. Furthermore, an imbalance of the antioxidant parameters was reported in some studies (Evans, 2000; Gonzalez-Duarte, 2012), where patients with adult GHD demonstrated a high degree of OS. Unfortunately, as of today only a small number of publications are dedicated to OS in GHD children, particularly to those subjected to treatment with rGH. In fact, we are aware of only one paper widely available (Mohn, 2005). This paper has demonstrated that OS parameters (index of susceptibility of low-density lipoprotein (LDL) to *in vitro* oxidation, malondialdehyde and vitamin E levels) in GHD children were substantially higher than those in the healthy control group, whereas after one year of rGH therapy these parameters returned to normal levels.

Therefore, in this study we evaluated several parameters of the blood antioxidant system: total antioxidant capacity (TAC) of plasma, activities of superoxide dismutase (SOD) and catalase, non-protein thiols (NT) and ceruloplasmin (CP) levels. These parameters can help to reveal OS in children with GHD and examine the effects of 12-month rGH therapy on the blood antioxidant system.

## Materials and Methods

Eleven treatment-naïve GH-deficient patients were included in this study (2 girls and 9 boys aged 3 to 9 years). The mean chronological age (CA) was  $6.1 \pm 2.2$  years, mean bone age (BA) was  $2.6 \pm 0.9$  years. None of them has ever undergone treatment with rGH.

The parameters of the blood antioxidant system were compared with those in a control group of 11 healthy prepubertal children (2 girls and 9 boys; aged 6 to 11 years; mean CA

9.3±1.4 years). The control group did not receive any placebo injections.

Standard deviation scores (SDS) for height and growth velocity were calculated using mean and standard deviation of the British reference population ~~from KIGS (Pfizer International Growth Database)~~ as described by Tanner (Tanner, 1966; Tanner, 1976). Pubertal stages are defined accordingly to Tanner (Tanner, 1962).

The SDS values for IGF-1 and IGFBP-3 were calculated for corresponding CA and gender ~~as described in Lofqvist et al.~~ (Lofqvist, 2001; Lofqvist, 2004).

All patients underwent a standard set of clinical and laboratory tests including physical and anthropometric evaluations, ~~x~~-x-rays of both hands and wrists ~~joints~~, as well as ~~an~~and-CT or MRI of the head. To verify the diagnosis, GH-provocation tests were performed: 5 samples were evaluated with clonidine (0, 30, 60, 90, and 120 minutes), and 7 samples were evaluated with insulin (0, 15, 30, 45, 60, 90, and 120 minutes). A value of less than 10 ng/ml was an argument in support of the GHD diagnosis (GH Research Society. Consensus guidelines, 2000). Peak values of GH response in both tests were 1.71±1.45 ng/ml (with the minimum and maximum values 0.13 and 3.5 accordingly). Idiopathic isolated GH-deficiency was diagnosed in all the cases studied. Clinical and biochemical blood analyses, as well as assays for IGF-1 and IGF-~~1~~binding protein-3 (IGFBP-3) were performed before ~~the~~ treatment and after 12 months ~~of into~~ rGH treatment. The IGF-1 levels were measured by immunoradiometric assay using a commercial IGF-1 RIA (Nichols Institute Diagnostics, Bad Nauheim); IGFBP-3 level was determined by enzyme immunoassay using commercial DSL-10-6600 ACTIVE™ IGFBP3 ELISA kit (DSL, Sinsheim, Germany). Daily ~~The~~ rGH injections were ~~was~~ subcutaneously administered daily in the evening. The daily dose of rGH was 0.033 mg per ~~1~~ kg ~~of~~ body weight ~~mass per day~~ (GH Research Society. Consensus guidelines, 2000).

Analyses of the antioxidant system were performed on whole blood samples, which were collected in the fasting state in the morning, ~~—~~ before ~~the treatment~~ and after 12 months of rGH treatment.

#### *Antioxidant Status Evaluation*

The blood antioxidant system was examined using a Hitachi 556 (Japan) spectrometer. The erythrocyte SOD activity was estimated by inhibition of epinephrine self-oxidation (Sun, 1978). The level of ceruloplasmin was estimated by measuring the enzymatic reaction with o-phenylenediamine (Brazhe, 2014). Catalase activity was measured in erythrocytes at 37°C according to Aebi (Aebi, 1984). Non-protein thiols were analyzed as described by Sedlak et

al. (Akhalaya, 2006). The total reducing power of antioxidants in the blood plasma was estimated by the ferric reducing ability of plasma (FRAP) assay, as described by Benzie and Strain (Benzie, 1996) with modifications: 350  $\mu$ L of distilled water was added to the test tube containing 3 mL of the reagent (working solution), then 50  $\mu$ L of plasma sample was added and mixed. After 10 minutes the samples were read at a wavelength of 593 nm. Photometric assay of the blood hemoglobin is based on the transformation of hemoglobin into its haemachrome form by sodium dodecyl sulphate, followed by absorption of the measuring light at 540 nm (Brazhe, 2014). Changes in the optical density were recorded with a Hitachi-556 spectrophotometer (Japan).

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### Statistics

The results were statistically processed using Statistica software, version 8.0. All data were normally distributed (Kolmogorov–Smirnov test,  $p < 0.05$ ). Statistical significance of differences for independent variables,  $P_1$  (between parameters of control group and parameters of case group before treatment) and  $P_2$  (between parameters of control group and parameters of case group after treatment), were calculated by one-way ANOVA with the post-hoc Tukey HSD test. The statistical significance of differences for dependent variables,  $P_3$  (between parameters of case group before and after rGH treatment) only for case group, was calculated by the paired T-test. Changes were considered significant at  $P < 0.05$ .

The power (one-tailed dependent and one-tailed independent T-test at  $\alpha = 0.05$  and the sample size of 11) was calculated using the Power Analysis box of Statistica 8.0.

### Ethics Statement

The study was approved by the Ethics Committee of the Endocrinology Research Centre, Moscow, Russian Federation (reference number: 14). Written informed consent was obtained from the patients and/or their parents or legal guardians.

## Results

### Anthropometric and Biochemical Parameters

Children treated with rGH demonstrated ~~an~~ increases in height, weight, height velocity, height SDS, and height velocity SDS (Table 1). To assess therapeutic efficacy, safety and compliance, IGF-1 SDS and IGFBP-3 SDS were assessed, which showed clear increases measured—their levels and SDSs also increased—during ~~the~~ rGH treatment.

Growth parameters after 12 months of rGH therapy significantly exceeded those at baseline

(see Table 1). After 12 months of treatment the patients' height was significantly increased ( $96.2 \pm 10.5$  versus  $108.0 \pm 10.8$  cm,  $P_3 = 0.000001$ ), ~~and which indicated linear growth during treatment. The~~ increase in growth velocity ( $3.4 \pm 1.2$  versus  $12.5 \pm 3.5$  cm/years,  $P_3 = 0.000013$ ) suggested that catch-up growth was significant. Thus, ~~the post-treatment~~ catch-up in growth was obvious: ~~differences were observed in~~ height SDS ~~increased from~~, ~~which was~~  $-3.6 \pm 0.9$  at the onset of therapy and ~~to increased significantly after 12 months of the treatment to~~  $-2.2 \pm 1.3$  ~~after 12 months~~ ( $P_3 = 0.000030$ ). ~~The levels of serum IGF-1 and IGFBP-3 also increased during treatment: the Serum SDS~~ IGF-1 SDS level was  $-3.1 \pm 1.8$  at baseline and increased to  $-0.7 \pm 1.9$  after 12 months of treatment ( $P_3 = 0.000689$ ); ~~the SDS~~ IGFBP-3 SDS ~~increased from was~~  $-4.0 \pm 3.5$  ~~to at the baseline, and increased to~~  $-0.2 \pm 1.7$  after treatment ( $P_3 = 0.002364$ ).

#### *The Antioxidant Status*

To evaluate the blood antioxidant status we selected the most characteristic parameters: total antioxidant capacity (TAC) of plasma measured by FRAP, superoxide dismutase (SOD) and catalase activities, non-protein thiols (NT) and ceruloplasmin levels. The FRAP is proportional to the reducing power of the main non-enzymatic antioxidants in the plasma, particularly uric and ascorbic acids. This parameter does not reflect the reduced glutathione and liposoluble antioxidants (e.g., vitamin E). Ceruloplasmin and SOD are responsible for both utilization of the superoxide anion radical and the regulation of variable-valence metal levels (copper and iron). Catalase and non-protein thiols play an important role in hydrogen peroxide utilization.

The parameters of blood antioxidant system (as mean  $\pm$  standard deviation) for control and case (before and after rGH treatment) groups ~~are~~ shown in Table 2. Before treatment, the TAC of plasma and the amount of NT were significantly lower than in the control group (Fig. 1):  $0.19 \pm 0.06$  versus  $0.26 \pm 0.07$  ~~a.u.~~ and  $0.70 \pm 0.22$  versus  $0.92 \pm 0.18$   $\mu\text{mol/ml}$  ( $P_1 = 0.0187$  and  $P_1 = 0.0319$ ), respectively. After rGH therapy only SOD activity differed from that in the control group ( $19.6 \pm 4.7$  versus  $14.5 \pm 3.4$  a.u./g Hb,  $P_2 = 0.0396$ ).

The paired T-test revealed no significant improvements in most of the parameters except for the TAC of plasma at the end of the 12-month treatment period (see Fig. 2). The TAC value increased significantly (by 30%) during treatment (from  $0.19 \pm 0.06$  ~~to until~~  $0.23 \pm 0.04$ ,  $P_3 = 0.0205$ ). The increase in NT level and SOD activity was not statistically significant ( $0.70 \pm 0.22$  versus  $0.78 \pm 0.15$   $\mu\text{mol/ml}$ ,  $P_3 = 0.3264$  and  $18.8 \pm 5.4$  versus  $19.6 \pm 4.7$  a.u./g Hb,  $P_3 = 0.7436$ , respectively), and ~~the~~ decrease in catalase activity and ceruloplasmin

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level during treatment were also not statistically significant ( $215 \pm 64$  versus  $190 \pm 43$  a.u./g Hb  $P_3=0.2257$  and  $581 \pm 100$  versus  $531 \pm 111$   $\mu\text{g/mL}$   $P_3=0.1061$ , respectively).

#### *Statistical Power*

The statistical power for all measured parameters was calculated (Table 3). The power varied greatly for different parameters. The highest power was revealed for anthropometric and biochemical parameters (height, weight, growth velocity, height SDS, growth velocity SDS, IGF-1, and IGFBP-3), which exceeded 0.91. For parameters of the blood antioxidant system, statistical power was considerably smaller. In this case, the most profound changes were found for TAC during GH treatment (0.82), and also in comparison of TAC, NT and SOD between the control and experimental groups (0.62, 0.56 and 0.57, respectively). The lowermost powers were found in changes in SOD and NT in the experimental group during rGH therapy (0.09 and 0.25, respectively) and also for the comparison of catalase and CP between the control and treatment groups (0.20 and 0.26, respectively).

#### **Discussion**

High power was found for anthropometric and biochemical parameters, in contrast to parameters of the antioxidant system. In general, we revealed ~~small~~ ~~inconsiderable~~ differences in the mean values of antioxidant system parameters and relatively high dispersion (standard deviation), which gives a rather high ~~type 2 error of the second kind~~ for the ~~small~~ sample volume of 11 persons. In other words, the main drawback in interpretation of the obtained results could be an incapacity to reveal significant differences due to low statistical power, even though they may be present. This is the reason why it is difficult to confidently reveal the absence of significant changes in parameters like NT, SOD, catalase and CP (power  $\leq 0.5$ ). Therefore, we have also considered non-significant differences between the parameters. However, in case of TAC changes during rGH treatment (0.82), and in comparing TAC, NT and SOD between control and experimental groups, the power of the tests was relatively high for a correct interpretation of the obtained results.

We also believe that some discrepancy in the age of patients between control and experimental groups is not crucial for an interpretation of our results because participants of both groups were prepubertal children with similar physiology and similar parameters of the antioxidant system. It is common practice in ~~studies~~ ~~researches~~ dedicated to the antioxidant status to include prepubertal children aged 2 to 11 years in a single group (see e.g. (Erden-

Inal, 2002; Singh, 2002; Llorente-Cantarero, 2013)).

We have demonstrated that parameters of the antioxidant system before ~~the~~ treatment are not balanced in GHD children: TAC and NT levels are lower, while SOD activity is elevated albeit statistically non-significantly.

The decreased TAC and NT level, the elevated SOD activity and the insignificantly raised level of ceruloplasmin registered in GHD children before rGH treatment, —all ~~this~~ indicates ~~the presence a development~~ of OS.

Obviously, a statistically significant resolution of only one parameter (TAC) during treatment indicates an insufficient prevention of the already ongoing OS in GHD children. However, a failure to reveal statically significant differences in TAC and NT (which characterize the total amount of antioxidants in the plasma and blood, respectively) after treatment between the groups, and also a non-significant decline in CP level (increase in CP level indicates inflammatory processes) still may suggest an improvement of the antioxidation state in children after therapy.

In our work we observed some evidence of blood antioxidant system improvements after rGH treatment, which is supported by the absence of a significant difference in TAC and NT after treatment between the control and treatment groups (using one-way ANOVA with post-hoc Tukey HSD test,  $p < 0.05$ ). Moreover, antioxidaton parameters such as TAC, NT and CP (but not SOD and catalase) ~~came~~ back to normal levels during treatment. Although these changes are small (~~not reaching statistical significance rather a tendency~~), we can conclude that these findings in general agree with data reported in Mohn et al. (Mohn, 2005), who showed an improvement in the antioxidant status, evidenced by a decline in free radicals in GHD children after rGH treatment.

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Parameters	Before GH therapy	After 12 months of GH therapy
Height, cm	96.2±10.5	108.0±10.8 P <sub>3</sub> =0.000001*
Weight, kg	15.2±3.9	17.8±5.5 P <sub>3</sub> =0.001118*
Growth velocity, cm/years	3.4±1.2	12.5±3.5 P <sub>3</sub> =0.000013*
Height SDS	-3.6±0.9	-2.2±1.3 P <sub>3</sub> =0.000030*
Growth velocity SDS	-3.2±1.8	7.4±3.9 P <sub>3</sub> =0.000026*
IGF-1 SDS	-3.1±1.8	-0.7±1.9 P <sub>3</sub> =0.000689*
IGFBP-3 SDS	-4.0±3.5	-0.2±1.7 P <sub>3</sub> =0.002364*

P<sub>3</sub> indicates the is-statistical significance between parameters of the case group before and after rGH treatment, ~~;- value was evaluated~~ using the paired T-test, p<0.05; (significant difference between data is marked by an asterisk).

Parameters	Control	Before GH therapy	After 12 months of GH therapy
Total antioxidant capacity of plasma, a.u.	0.26±0.07	0.19±0.06 P <sub>1</sub> =0.0187*	0.23±0.04 P <sub>2</sub> =0.5967 P <sub>3</sub> =0.0205*
Non-Protein Thiols, µmol/ml	0.92±0.18	0.70±0.22 P <sub>1</sub> =0.0319*	0.78±0.15 P <sub>2</sub> =0.2146 P <sub>3</sub> =0.3264
Superoxide Dismutase (SOD), a.u./g Hb	14.5±3.4	18.8±5.4 P <sub>1</sub> =0.0958	19.6±4.7 P <sub>2</sub> =0.0396* P <sub>3</sub> =0.7436
Catalase, a.u./gHb	216±38	215±64 P <sub>1</sub> =0.9991	190±43 P <sub>2</sub> =0.4506 P <sub>3</sub> =0.2257
Ceruloplasmin, µg/mL	518±70	581±100 P <sub>1</sub> =0.2866	531±111 P <sub>2</sub> =0.9518 P <sub>3</sub> =0.1061

P<sub>1</sub> indicatess the statistical significance between parameters of the control group and those of the case group before treatment, P<sub>2</sub> is the statistical significance between parameters of the control group and those of the of-case group after treatment; values wereas evaluated using the one-way ANOVA with post-hoc Tukey HSD test, p<0.05. P<sub>3</sub> is the statistical significance between parameters of case group before and after rGH treatment, ~~;- value was evaluated~~ using the paired T-test, p<0.05. Significant differences between data areis marked by an asterisk.

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Parameter	Power
Dependent data (one-tailed dependent T-test $\alpha=0.05$ , the sample size - 11)	
Height, cm	1.00
Weight, kg	0.99
Growth velocity	1.00
Height SDS	1.00
Growth velocity SDS	1.00
IGF-1, nMol/L	0.92
IGFBP-3, nMol/L	1.00
IGF-1 SDS	1.00
IGFBP-3 SDS	0.98
Total antioxidant capacity of plasma, a.u.	0.82
Non-Protein Thiols, $\mu\text{mol/ml}$	0.25
Superoxide Dismutase (SOD), a.u./g Hb	0.09
Catalase, a.u./g Hb	0.33
Ceruloplasmin, $\mu\text{g/mL}$	0.50
Independent data (one-way ANOVA $\alpha=0.05$ , the sample size - 11)	
Total antioxidant capacity of plasma, a.u.	0.62
Non-Protein Thiols, $\mu\text{mol/ml}$	0.56
Superoxide Dismutase (SOD), a.u./g Hb	0.57
Catalase, a.u./g Hb	0.20
Ceruloplasmin, $\mu\text{g/mL}$	0.26