Imbalance in the blood antioxidant system in growth hormone-deficient children before and after 1 year of recombinant growth hormone therapy

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The aim of our study was to examine the effects of 12-month therapy with recombinant growth hormone (rGH) on the blood antioxidant system in children with growth hormone deficiency (GHD). Total antioxidant capacity (TAC) of plasma was measured by FRAP (ferric reducing antioxidant power or ferric reducing ability of plasma); activities of superoxide dismutase (SOD) and catalase (CAT) in erythrocytes were assessed; non-protein thiols (NT) and ceruloplasmin (CP) levels were also measured. These parameters were determined before and after 12 month of rGH treatment. Eleven treatment-naive prepubertal children with growth hormone deficiency were included in the study. Another 11 prepubertal children comprised a control group. Before rGH treatment TAC of plasma and NT level in the control group were significantly lower (726±196 vs. 525±166 µmol/L, P=0.0182 and 0.92±0.18 vs. 0.70±0.22 µmol/ml, P=0.0319, before and after the therapy, respectively). The only parameter that significantly (19.6±4.7 vs 14.5±3.4 Units/g Hb, P=0.0396) exceeded the same in the control group after rGH therapy was SOD activity. However, none of the measured parameters of antioxidant system in GHD children, except for TAC (525±166 vs. 658±115 µmol/L, P=0.0205), exhibited significant improvement toward the end of the 12-month treatment period, although non-significant changes in CAT activity and CP level were also observed. This work has demonstrated that some parameters of the blood antioxidant system are out of balance and even impaired in GHD children. A 12-month treatment with rGH resulted in a partial improvement of the antioxidant system.

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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 those subjected to treatment with rGH. In fact, we are aware of only one paper (Mohn, 2005). This paper 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-naive 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 ± 2.2 years, mean bone age (BA) was 2.6 ± 0.9 years. None of them has ever undergone treatment with rGH.

45   The parameters of the blood antioxidant system were compared with those in a control

46 group of 11 healthy prepubertal children (2 girls and 9 boys; aged 6 to 11 years; mean CA

47 9.3±1.4 years). The control group did not receive any placebo injections.

48   Standard deviation scores (SDS) for height and growth velocity were calculated using

49 mean and standard deviation of the British reference population as described by Tanner (Tanner,

50 1966; Tanner, 1976). Pubertal stages are defined accordingly to Tanner (Tanner, 1962).

51   The SDS values for IGF-1 and IGFBP-3 were calculated for corresponding CA and

52 gender (Lofqvist, 2001; Lofqvist, 2004).

53   All patients underwent a standard set of clinical and laboratory tests including physical

54 and anthropometric evaluations, x-rays of both hands and wrists, as well as anCT or MRI of the

55 head. To verify the diagnosis, GH-provocation tests were performed: 5 samples were evaluated

56 with clonidine (0, 30, 60, 90, and 120 minutes), and 7 samples were evaluated with insulin (0,

57 15, 30, 45, 60, 90, and 120 minutes). A value of less than 10 ng/ml was an argument in support

58 of the GHD diagnosis (GH Research Society. Consensus guidelines, 2000). Peak values of GH

59 response in both tests were 1.71±1.45 ng/ml (with the minimum and maximum values 0.13 and

60 3.5 accordingly). Idiopathic isolated GH-deficiency was diagnosed in all the cases studied.

61 Clinical and biochemical blood analyses, as well as assays for IGF-1 and IGF binding protein-3

62 (IGFBP-3) were performed before treatment and after 12 months of rGH treatment. The IGF-1

63 levels were measured by immunoradiometric assay using a commercial IGF-1 RIA (Nichols

64 Institute Diagnostics, Bad Nauheim); IGFBP-3 level was determined by enzyme immunoassay

65 using commercial DSL-10-6600 ACTIVE™ IGFBP3 ELISA kit (DSL, Sinsheim, Germany).

66 Daily rGH injections were subcutaneously administered in the evening. The daily dose of rGH

67 was 0.033 mg per kg body weight (GH Research Society. Consensus guidelines, 2000).

68   Analyses of the antioxidant system were performed on whole blood samples, which were

69 collected in the fasting state in the morning, before and after 12 months of rGH treatment.

70 *Antioxidant Status Evaluation*

71 The erythrocyte SOD activity was estimated by inhibition of epinephrine self-oxidation at 25°C

72 (Sun, 1978). One unit of SOD activity was defined as that amount of SOD required to cause

73 50% inhibition of the oxidation of the epinephrine. The SOD activity was expressed as units per

74 gram of haemoglobin (Units/g Hb).

75 The level of ceruloplasmin was estimated by measuring the enzymatic reaction with o-
76 phenylenediamine (Brazhe, 2014).

77 Catalase activity was measured in erythrocytes at 37°C according to Aebi (Aebi, 1984). One
78 unit of catalase activity (k) was defined as the amount of enzyme that degrades one mmol
79 H_2O_2/min at initial concentration of 10 mmol/L at pH 7.0. The catalase activity was expressed as
80 the unit per gram hemoglobin (k/g Hb).

81 Non-protein thiols were analyzed as described by Sedlak et al. (Akhalaya, 2006).

82 The TAC in the blood plasma was estimated by the ferric reducing ability of plasma (FRAP)
83 assay, as described by Benzie and Strain (Benzie, 1996) with modifications: 350 µL of distilled
84 water was added to the test tube containing 3 mL of the reagent (working solution), then 50 µL
85 of plasma sample was added and mixed. After 10 minutes the samples were read at a
86 wavelength of 593 nm. The method is based on the reduction of colorless ferric (Fe^{3+})
87 tripyridyltriazine complex in working solution to blue colored ferrous (Fe^{2+}) tripyridyltriazine
88 complex at low pH. The TAC values were obtained by comparing the absorption change in the
89 test mixture with those obtained from increasing concentrations of Fe^{2+} and expressed as µmol
90 of Fe^{2+} equivalents per L of sample.

91 Photometric assay of the blood hemoglobin is based on the transformation of hemoglobin into
92 its haemachrome form by sodium dodecyl sulphate, followed by absorption of the measuring
93 light at 540 nm (Brazhe, 2014).

94 Changes in the optical density were recorded with a Hitachi-556 spectrophotometer (Japan).

95 *Statistics*
96 The results were statistically processed using Statistica software, version 8.0. All data were
97 normally distributed (Kolmogorov–Smirnov test, $p<0.05$). Statistical significance of differences
98 for independent variables, P_1 (between parameters of control group and parameters of case
99 group before treatment) and P_2 (between parameters of control group and parameters of case

100 group after treatment), were calculated by one-way ANOVA with the post-hoc Tukey HSD test.

101 The statistical significance of differences for dependent variables, P_3 (between parameters of

102 case group before and after rGH treatment) only for case group, was calculated by the paired T-

103 test. Changes were considered significant at P<0.05.

104 The power (one-tailed dependent and one-tailed independent T-test at α=0.05 and the sample

105 size of 11) was calculated using the Power Analysis box of Statistica 8.0.

106 *Ethics Statement*

107 The study was approved by the Ethics Committee of the Endocrinology Research Centre,

108 Moscow, Russian Federation (reference number: 14). Written informed consent was obtained

109 from the patients and/or their parents or legal guardians.

110
111 **Results**

112 *Anthropometric and Biochemical Parameters*

113 Children treated with rGH demonstrated increases in height, weight, height velocity, height

114 SDS, and height velocity SDS (Table 1). To assess therapeutic efficacy, safety and compliance,

115 IGF-1 SDS and IGFBP-3 SDS were assessed, which showed clear increases during rGH

116 treatment.

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

118 (Table 1). After 12 months of treatment the patients' height was significantly increased

119 (96.2±10.5 versus 108.0±10.8 cm, P_3=0.000001), and the increase in growth velocity (3.4±1.2

120 versus 12.5±3.5 cm/years, P_3=0.000013) suggested that catch-up growth was significant. Thus,

121 catch-up in growth was obvious: height SDS increased from -3.6±0.9 at the onset of therapy and

122 to -2.2±1.3 after 12 months (P_3=0.000030). Serum IGF-1 SDS level was -3.1±1.8 at baseline

123 and increased to -0.7±1.9 after 12 months of treatment (P_3=0.000689); IGFBP-3 SDS increased

124 from -4.0±3.5 to -0.2±1.7 after treatment (P_3=0.002364).

125 *The Antioxidant Status*

126 To evaluate the blood antioxidant status we selected the most characteristic parameters:

127 total antioxidant capacity (TAC) of plasma measured by FRAP, superoxide dismutase (SOD)

128 and catalase activities, non-protein thiols (NT) and ceruloplasmin levels. The FRAP is

129 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 ± 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): 525±166 versus 726±196 μmol/L and 0.70±0.22 versus 0.92±0.18 μmol/ml (P_1=0.0182 and P_1=0.0319), respectively. After rGH therapy only SOD activity differed from that in the control group (19.6±4.7 versus 14.5±3.4Units/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 525±166 to 658±115, P_3=0.0205). The increase in NT level and SOD activity was not statistically significant (0.70±0.22 versus 0.78±0.15 μmol/ml, P_3=0.3264 and 18.8±5.4 versus 19.6±4.7Units/g Hb, P_3=0.7436, respectively), and the decrease in catalase activity and ceruloplasmin level during treatment were also not statistically significant (215±64 versus 190±43 k/g Hb P_3=0.2257 and 581±100 versus 531±111 μg/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

160

161   High power was found for anthropometric and biochemical parameters, in contrast to

162   parameters of the antioxidant system. In general, we revealed small differences in the mean

163   values of antioxidant system parameters and relatively high dispersion (standard deviation),

164   which gives a rather high type 2 error for the small sample volume of 11 persons. In other

165   words, the main drawback in interpretation of the obtained results could be an incapacity to

166   reveal significant differences due to low statistical power, even thought they may be present.

167   This is the reason why it is difficult to confidently reveal the absence of significant changes in

168   parameters like NT, SOD, catalase and CP (power ≤ 0.5). Therefore, we have also considered

169   non-significant differences between the parameters. However, in case of TAC changes during

170   rGH treatment (0.82), and in comparing TAC, NT and SOD between control and experimental

171   groups, the power of the tests was relatively high for a correct interpretation of the obtained

172   results.

173   We also believe that some discrepancy in the age of patients between control and

174   experimental groups is not crucial for an interpretation of our results because participants of both

175   groups were prepubertal children with similar physiology and similar parameters of the

176   antioxidant system. It is common practice in studies dedicated to the antioxidant status to

177   include prepubertal children aged 2 to 11 years in a single group (see e.g. (Erden-Inal, 2002;

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

179   We have demonstrated that parameters of the antioxidant system before treatment are not

180   balanced in GHD children: TAC and NT levels are lower, while SOD activity is elevated albeit

181   statistically non-significantly. The decreased TAC and NT level, the elevated SOD activity and

182   the insignificantly raised level of ceruloplasmin registered in GHD children before rGH

183   treatment, all indicate the presence of OS.

184   Obviously, a statistically significant resolution of only one parameter (TAC) during

185   treatment indicates an insufficient prevention of the already ongoing OS in GHD children.

186   However, a failure to reveal statically significant differences in TAC and NT (which

187   characterize the total amount of antioxidants in the plasma and blood, respectively) after

188   treatment between the groups, and also a non-significant decline in CP level (increase in CP

189   level indicates inflammatory processes) still may suggest an improvement of the antioxidation

190   state in children after therapy.

191   In our work we observed some evidence of blood antioxidant system improvements after

192   rGH treatment, which is supported by the absence of a significant difference in TAC and NT

193   after treatment between the control and treatment groups (using one-way ANOVA with post-hoc

194   Tukey HSD test, $p<0.05$). Moreover, antioxidaton parameters such as TAC, NT and CP (but not

195   SOD and catalase) came back to normal levels during treatment. Although these changes are

196   small (not reaching statistical significance), we can conclude that these findings in general agree

197   with data reported in Mohn et al. (Mohn, 2005), who showed an improvement in the antioxidant

198   status, evidenced by a decline in free radicals in GHD children after rGH treatment.

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Figure 1: Changes in TAC (*a*), NT level (*b*), erythrocyte SOD (*c*) and catalase (*d*) activities, CP level (*e*) after a 12 month rGH treatment (case-control data).

The data presented as a standard box and whisker plot (where the length of the box is an interquartile range) with the mean (closed square) and experimental data (open circle), the ends of the whiskers represent the maximal and minimal values. In the picture data of the control group (black box) and that of the case group before (red box) and after (blue box) rGH treatment is shown. P_1 is the statistical significance between parameters of the control group and those of the case group before treatment; P_2 is the statistical significance between parameters of the control group and those of the of case group after treatment. The experimental data were evaluated by the one-way ANOVA with post-hoc Tukey HSD test. The significant difference ($P<0.05$) for P_1 and P_2 is shown by black rectangles.
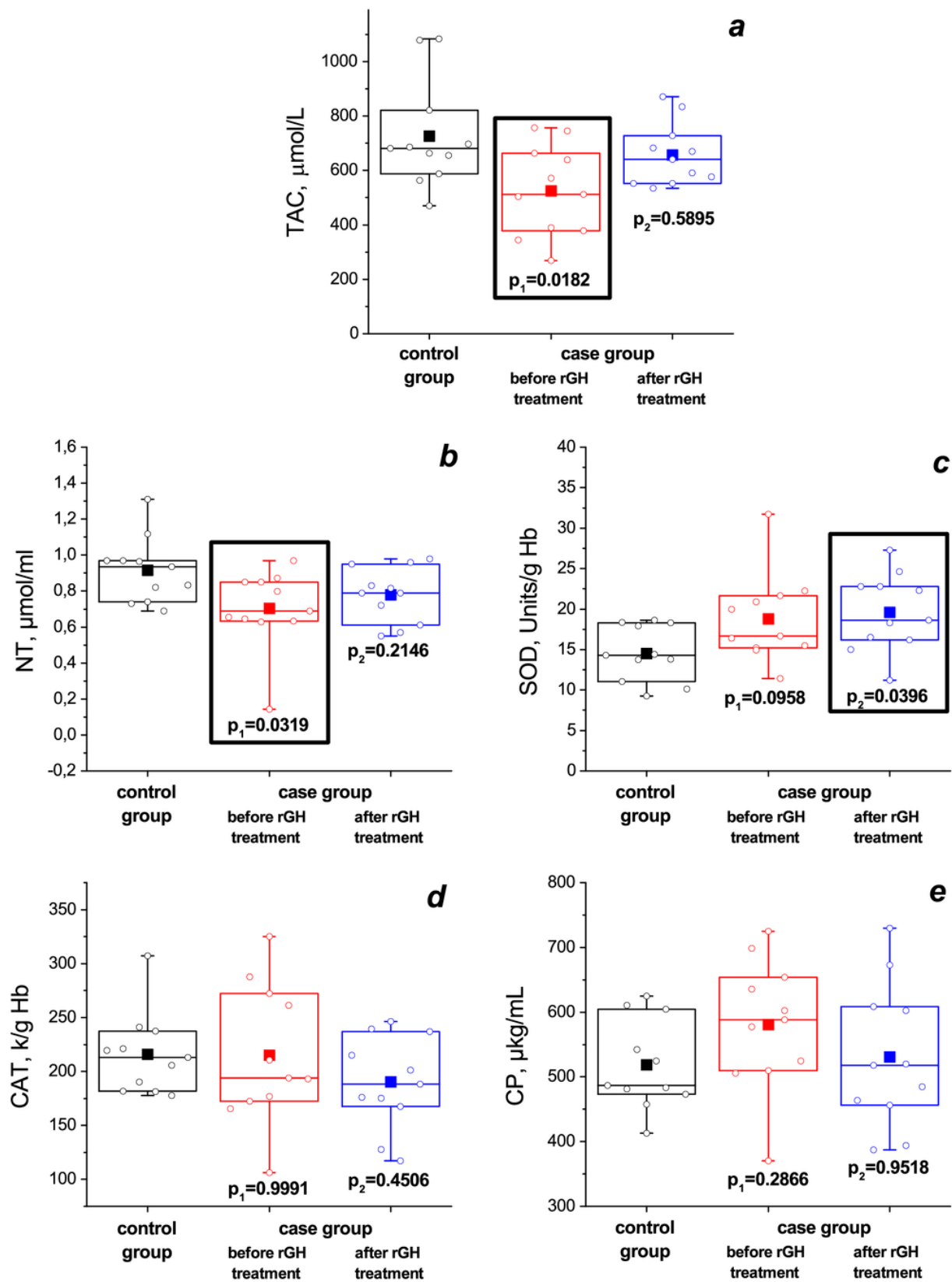
Figure 2: Changes in TAC (*a*), NT level (*b*), erythrocyte SOD (*c*) and catalase (*d*) activities, CP level (*e*) during the 12 month rGH treatment (longitudinal data).

The data of case group before and after rGH treatment is shown in picture. Different symbols corresponds to the parameters obtained in various patients before and after the treatment (data of each patient is connected by line). The mean values ± standard deviations for case group before and after treatment is shown as open red circles with whiskers. P_3 is the statistical significance between parameters of case group before and after rGH treatment. The experimental parameters were evaluated by the paired T-test, $p < 0.05$. Significant difference between data is marked by an asterisk.
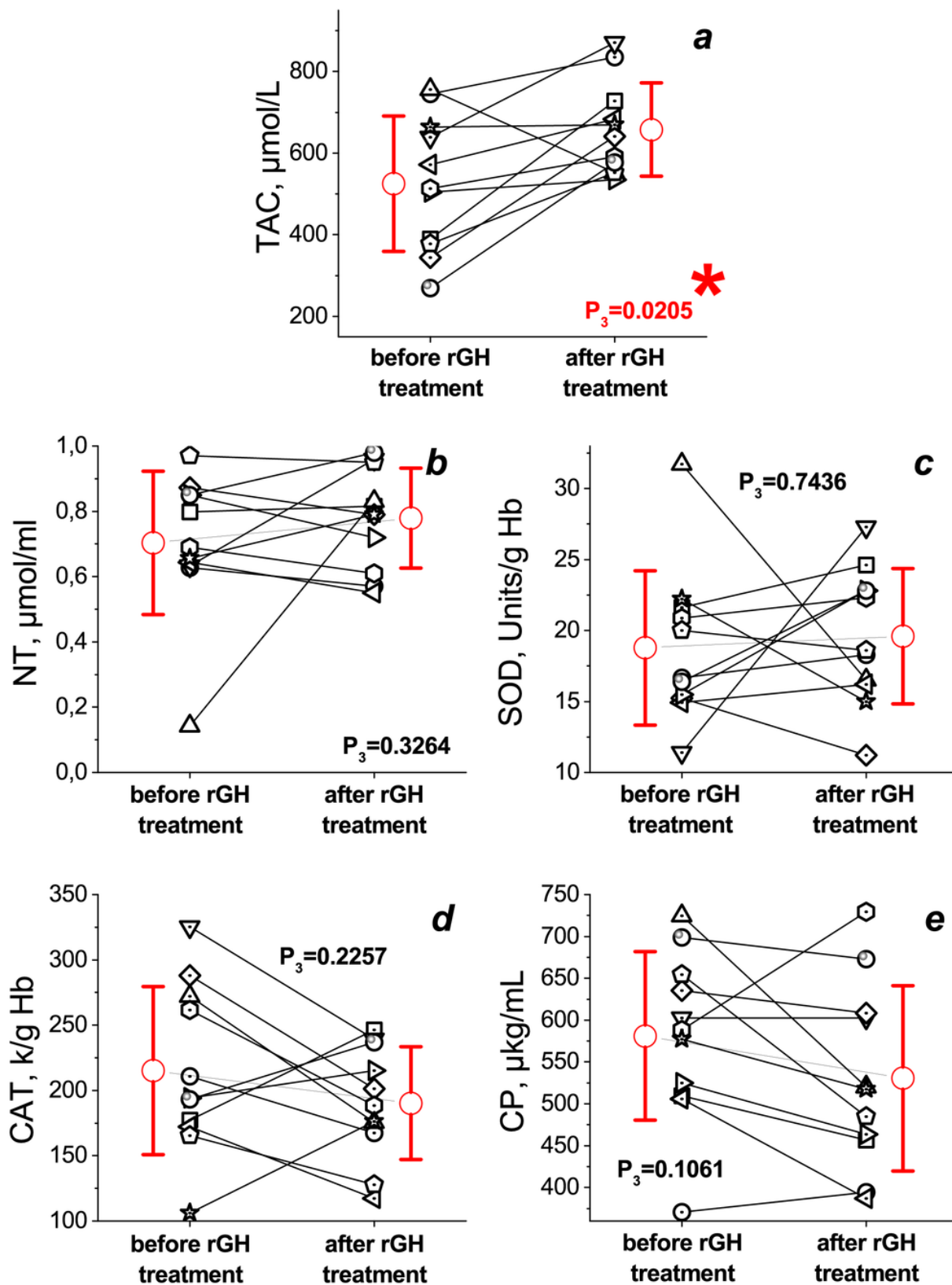
Table 1: Effect of growth hormone therapy on anthropometric and biochemical parameters of GHD children.

Parameters	Before GH therapy	After 12 months of GH therapy
Height, cm	96.2±10.5	108.0±10.8 P_3=0.000001*
Weight, kg	15.2±3.9	17.8±5.5 P_3=0.001118*
Growth velocity, cm/years	3.4±1.2	12.5±3.5 P_3=0.000013*
Height SDS	-3.6±0.9	-2.2±1.3 P_3=0.000030*
Growth velocity SDS	-3.2±1.8	7.4±3.9 P_3=0,000026*
IGF-1 SDS	-3.1±1.8	-0.7±1.9 P_3=0.000689*
IGFBP-3 SDS	-4.0±3.5	-0.2±1.7 P_3=0.002364*

P_3 indicates the statistical significance between parameters of the case group before and after rGH treatment, using the paired T-test, P<0.05 (significant difference between data is marked by an asterisk).

Table 2: Effect of growth hormone therapy on parameters of the blood antioxidant status of GHD children.

Parameters	Control	Before GH therapy	After 12 months of GH therapy
Total antioxidant capacity of plasma, μmol/L	726±196	525±166 P_1=0.0182*	658±115 P_2=0.5895 P_3=0.0205*
Non-Protein Thiols, μmol/ml	0.92±0.18	0.70±0.22 P_1=0.0319*	0.78±0.15 P_2=0.2146 P_3=0.3264
Superoxide Dismutase (SOD), Units/g Hb	14.5±3.4	18.8±5.4 P_1=0.0958	19.6±4.7 P_2=0.0396* P_3=0.7436
Catalase, k/g Hb	216±38	215±64 P_1=0.9991	190±43 P_2=0.4506 P_3=0.2257
Ceruloplasmin, μg/mL	518±70	581±100 P_1=0.2866	531±111 P_2=0.9518 P_3=0.1061

P_1 indicates the statistical significance between parameters of the control group and those of the case group before treatment, P_2 is the statistical significance between parameters of the control group and those of the case group after treatment; values were evaluated using the one-way ANOVA with post-hoc Tukey HSD test, $p<0.05$. P_3 is the statistical significance between parameters of case group before and after rGH treatment, using the paired T-test, $p<0.05$. Significant differences between data are marked by an asterisk.

Table 3: The calculated values of statistic power.

Parameter	Power
Dependent data (one-tailed dependent T-test α=0.05, the sample size - 11)	

Parameter	Power
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, µmol/L	0.82
Non-Protein Thiols, µmol/ml	0.25
Superoxide Dismutase (SOD), Units/g Hb	0.09
Catalase, k/g Hb	0.33
Ceruloplasmin, µg/mL	0.50

Independent data (one-way ANOVA α=0.05, the sample size - 11)

Parameter	Power
Total antioxidant capacity of plasma, µmol/L	0.62
Non-Protein Thiols, µmol/ml	0.56
Superoxide Dismutase (SOD), Units/g Hb	0.57
Catalase, k/g Hb	0.20
Ceruloplasmin, µg/mL	0.26