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The synergistic action of antioxidative enzymes – correlations of catalase and superoxide dismutase in the development and during the treatment of type 2 diabetes

Узајамно деловање антиоксидативних ензима – корелациони односи каталазе и супероксид-дисмутазе током развоја и лечења дијабетеса типа 2

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Узајамно деловање антиоксидативних ензима – корелациони односи каталазе и супероксид-дисмутазе током развоја и лечења дијабетеса типа 2

SUMMARY

Introduction/Objective The wider literature review of analysis in levels of Catalase (CAT) or Superoxid dismutase (SOD) enzymes in type 2 diabetes mellitus (T2DM) patients shows no pronounced consistency. We have assumed that the onset of diabetes does not significantly change individual quantities of these enzymes, but instead it changes the relationship of these enzymes.

Methods: The study consisted of 4 groups (n = 30 for each group): obese individuals with disturbed glucose metabolism (subjects with newly diagnosed type 2 diabetes (T2DM)) before and after metformin treatment initiation, obese subjects with normal glucose tolerance (NGT) and a control group of healthy normal weight subjects. Appropriate anthropometric measurements and laboratory tests of biochemical parameters and antioxidative enzymes were carried out in all participants.

Results Our study has confirmed that correlation of enzymes CAT and SOD is significantly changed in patients with newly diagnosed T2DM, and that it can be restored by reestablishment of glucose homeostasis with adequate antidiabetic treatment.

Conclusion The applied therapy restores the dynamic balance of CAT and SOD, mainly through the reintegration of the new equilibrium in the enzyme system after achieving better glycemic control. These conclusions are only valid in the initial stages of T2DM treatment.

Keywords: antioxidative enzymes, obesity, glucose metabolism, metformin

САЖЕТАК

Увод/Циљ Литературни преглед појединачних анализа вредности ензима каталазе (КАТ) или супероксид дисмутазе (СОД) код оболелих од дијабетеса типа 2 нема изражену конзистентност. Пошли смо од претпоставке да се при појави дијабетеса не мењају значајно појединачни квантитети наведених ензима, али да се значајно нарушава однос наведених ензима.

Методе рада: Студија се састојала од 4 групе (n = 30 за сваку групу): гојазних особа са поремећеним метаболизмом глукозе (субјекти са новодијагностикованим тип 2 дијабетесом) пре и у току метформинске терапије, гојазних субјеката са нормалном толеранцијом глукозе (НТГ) и контролне групе здравих нормалне телесне масе. Одговарајућа антропометријска мерења и лабораторијска испитивања биохемијских параметара и антиоксидативних ензима су спроведена код свих учесника.

Резултати Наша студија доказује значајне промене вредности линеарних корелација ензима каталазе и супероксид дисмутазе код болесника са новооткривеним дијабетесом типа 2, и може се вратити на ниво гојазних болесника пре појаве дијабетеса уз адекватну антидијабетску терапију.

Закључак: Примењена терапија метформином враћа динамички баланс ензима КАТ и СОД на ниво ензима гојазних болесника са нормалном гликорегулацијом, кроз реинтеграцију новог равнотежног система вредности ензима и расподела вредности ензима. Ови закључци важе само за почетне фазе лечења дијабетеса типа 2.

Кључне речи: антиоксидативни ензими; гојазност; метаболизам глукозе; метформин

INTRODUCTION

Oxidative stress is one of the important factors contributing to the pathogenesis of the large number of diseases such as obesity, diabetes, atherosclerosis, inflammatory, malignant and certain neurodegenerative diseases [1, 2]. The enzyme superoxide dismutase (SOD) protects the cells from superoxide anion radicals entering into the chemical reaction and turning these radicals into hydrogen peroxide, which is further detoxified to H₂O in the lysosomes

through the enzyme Catalase (CAT), or in the mitochondria through the enzyme glutathione peroxidase (Gpx) [3].

Hyperglycemia [4], increased intake of free fatty acids and excessive exposure to ultraviolet radiation are leading to increased oxidative stress, but the role of antioxidant enzymes is still not fully clarified [5]. Previous studies came to conflicting results regarding the activities of these antioxidant enzymes in diabetic patients. Levels of SOD in diabetic patients were found to be significantly elevated [6], significantly reduced [7], or unchanged in comparison to the control group [8]. Similarly, other authors concluded that CAT level is significantly higher [9], significantly lower [10] or is the same as in individuals from the control group [11].

For the purpose of clarifying the role of some of antioxidant enzymes, we studied the functional association between glycemia and CAT and SOD function. Although number of previous studies already assessed levels of CAT and SOD in type 2 diabetes (T2DM) patients, correlation of these two enzymes has rarely been the subject of previous researches. The principal aim of this study is to examine functional association of these two enzymes, which may be crucial for the optimal antioxidative protection achievement. Secondary, this study aims to evaluate the possible influence of the metformin therapy on these enzymes in diabetic patients.

METHODS

Study is conducted at Clinic for Endocrinology, Diabetes and Metabolic Disorders, Clinical Center of Vojvodina, Novi Sad, Serbia and it enrolled 90 age and sex matched individuals who gave their written consent prior to participation in the study. Study has been carried out in accordance with Helsinki declaration and it was done in accord with standards of the institutional Committee on Ethics.

Body mass index (BMI) was calculated as weight (kg) divided by height squared (m) (kg/m^2). We excluded individuals with all chronic conditions that affect the oxidative status of the organism.

Subjects were divided in the following groups:

- 1) 30 obese individuals with normal glucose tolerance and normal distribution of BMI ($37.37 \pm 6.11 \text{ kg/m}^2$);
- 2) 30 obese individuals with newly diagnosed T2DM and normal distribution of BMI on the baseline ($34.41 \pm 4.68 \text{ kg/m}^2$) analyzed before and during metformin therapy;
- 3) 30 healthy normal weight control individuals with a normal distribution of BMI ($23.34 \pm 3.12 \text{ kg/m}^2$);

The values of glycemia, CAT and SOD in group 2 were analysed before and three months after the initiation of metformin therapy (1000 mg per day in all subjects).

Blood was sampled for the analysis of various parameters. Fasting and two hour postprandial glucose were determined by enzymatic methods. Determination of parameters of oxidative stress was performed after the following preparation of the blood sample: 0.5 mL of heparinized blood was centrifuged for ten minutes at 3000 rotations per minute. After the plasma separation, red blood cells were washed four times with 3 mL of saline followed by stirring and centrifuging for ten minutes at 3000 rotations per minute. Washed red blood cells were supplemented with 2 mL of distilled water. The obtained hemolysate was divided into two samples for analysis of CAT and SOD in red blood cells. The analysis of CAT was carried out by monitoring of the fall in absorbance at 240 nm in a solution of hydrogen peroxide with phosphate buffer. The obtained values were expressed as U/mL. *The minus sign ahead of the value was (can be) ignored.* The analysis of SOD of red blood cells was performed by enzymatic kinetic method using commercial BIOREX kit. The obtained values were expressed as U/g of hemoglobin.

RESULTS

Statistical analysis of parameters and distribution SOD, CAT and blood glucose

In Table 1 values are given (mean value and standard deviation) for parameters SOD, CAT, fasting and postprandial 2h glucose (G0H and G2H) in study groups.

For the established normal distributions of SOD in the control group and in the group of obese individuals, t-test confirms a significant difference in mean values of this enzyme ($t = 3.53$, $p = 0.0014$). Uniform distribution in T2DM group refers to the systematic change of SOD after the treatment.

For the established normal distributions of CAT in the control group, the group of obese individuals and in the T2DM group before the treatment, using t-test finds no significant changes in the mean value of CAT.

Significant change in the structure of distribution of CAT in the T2DM group after the treatment also refers to the systematic change and to absolute differences in CAT with complementary groups. This difference is caused by treatment.

For the established normal distributions of G0H in the control group and in the group of and obese individuals, using t-test confirms a significant difference in mean values of G2H ($t = -3.09$, $p = 0.0044$).

The difference between mean values of G0H ($Z = 4.31$; $p = 0.000016 < 0.05$) and G2H ($Z = 3.31$; $p = 0.000856 < 0.05$) in the T2DM group before and after the treatment is significant (Signum test). Therapy had a significant effect.

Correlations of SOD and CAT

In order to determine the nature of the oxidative stress, the starting point was the relationship between SOD as the independent variable, and CAT as the dependent variable. The following equations of linear regression and correlation coefficients were established and given in Figure 4.

It should be noted that analytical parameters of the regression line, i.e. free member, and the coefficient for the independent variable are very close (-160.6 for control group and -163.4 for obese individuals).

However, in the group of patients with newly diagnosed diabetes systematic change in the relation between SOD and CAT is occurring. The value of the free member (-90.07) changes drastically for about 44% (from initial ≈ -160) while the coefficient for the independent variable changes the sign (-0.1782). Dependent systematic change describes and validates the negative correlation coefficient ($r = -0.5461$).

The applied therapy in patients with diabetes returns the correlation parameters of SOD and CAT close to established among the control group and the group of obese individuals. The value of the free member of the regression line (-162.1) is close to the value in the control

group and in the group of obese individuals (≈ -160), so therapy eliminated difference of 44%, which was the result of the diabetes onset. The coefficient of the independent variable is positive again (+0.1389). The value of correlation coefficient of the enzyme after the treatment ($r = +0.2503$) points out that "moderate" systematic association in diabetes is reduced to "low" after the treatment.

The biggest difference in changes in values of correlation coefficients occurs in the group of T2DM patients before and after the treatment. Pearson's test declares this change as significant $p = 0.0011$, i.e. therapy significantly changes linear relationships of SOD and CAT. A more detailed insight into the effect of therapy linear regressions of SOD and CAT in T2DM group before and after the treatment is given in Figure 5. Directly proportional relationships are more pronounced in SOD ($r = +0.3812$) than in CAT ($r = +0.2977$).

Low values of coefficients of the variable before the treatment (0.20164 for SOD and 0.26791 for CAT) (Figure 5), are excluding the possibility of the individual intense change in the value of enzymes caused by therapy.

Determining the interval of synergistic influence of SOD and CAT on blood glucose levels

Specific and significant change in the correlations between SOD and CAT prior to the treatment, intrigues further analysis of paths through which enzymes influence the basic parameter of diabetes – blood glucose. The analysis continues with exploring of synergic influence of SOD and CAT primarily on the G0H. From statistical data the approximate two-dimensional function of independent variables (SOD and CAT) and dependent variable (G0H) is formed, which is shown in Figure 6. The onset of diabetes in obese individuals, in addition to drastic changes of coefficients in linear regression line equation of the relationship between SOD and CAT, the change of two-dimensional G0H function is expressed. The appearance of the glucose maximum on coordinates of independent variables $SOD \approx 150$ and $CAT \approx -110$ marked by values of dependent variable $G0H > 10$.

After treatment period, mild parabola determined by SOD with the minimum line at about $SOD \approx 250$ which is in linear decrease in the function of CAT emerges. It is evident that the treatment is "calming" the maximum and that the inflection limit for CAT is ≈ -135 (underlined in Figure 6).

When forming two complementary groups of T2DM patients before and after the treatment with regard to cutoff point of $CAT \approx -135$, the group with $0 > CAT > -135$ have mean G0H of 10.969, while the group with $-135 > CAT > -\infty$ have mean G0H of 7.883. These values differ significantly based on analysis of variance (ANOVA) $p = 0.03537 < 0.05$ ($df = 23, F = 4.9977$).

The equation of the linear regression of SOD and CAT ($CAT = -90.07 - 0.1782 \times SOD$) can be expressed in an inverse form in which CAT is independent variable and SOD is dependent variable. If we enter characteristic value of $CAT = -135$ in this linear regression equation, the assumed characteristic value SOD is obtained:

$$SOD = 48.33 - 1.674 \times CAT \Rightarrow SOD = 48.33 - 1.674 \times -135 = 274.32 \approx 270$$

Adopted value $SOD = 270$ is characteristic because it represents the approximate direction of propagation of minimum parabolic drawing, the axis of the two-dimensional function of G0H dependence in T2DM patients Diabetes type 2 (Figure 6, after the treatment).

However, although among low values SOD does not give a significant difference in G0H value by itself, a synergistic effect of factor ($0 < SOD < 270$) with factor ($0 > CAT > -135$) reveals a group of patients which has an exceptionally high G0H before the treatment with the mean value of $G0H = 12.656$ in contrast to the value of complementary group $G0H = 7.7063$. The analysis of variance for the declared intervals of value of the intervals of both enzymes (MANOVA test) points out the significant difference between groups with the significance threshold of $p = 0.000424 < 0.01 < 0.05$.

In search for a possible dynamic of influence of SOD and CAT factors on blood glucose levels, analysis of two-dimensional dependence of G2H in a function of independent variables (SOD and CAT) is continued. Graphics of approximate function for T2DM patients before and after the treatment are given in Figure 7. After the treatment, changes are occurring for a variable G2H - from the parabolic form (Figure 6) to the translation of maximum before the treatment into zones of high values of SOD, which are highlighted by arrows in Figure 7. However, these changes are not significant (MANOVA $p = 0.0797 < 0.05!$).

DISCUSSION

The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) constitute a first line antioxidant defense system which plays a key role in the defense mechanisms in biological systems [12]. Controversial reports on changes in serum antioxidative enzyme activity of T2DM patients have been published [13]. Our study demonstrated very specific changes in value and distribution of CAT and SOD. CAT has proven to be more stable enzyme which did not change the systematic distribution with the onset of diabetes. The mean value of CAT after the onset of diabetes remained at the level similar to one in the control group and in the group of obese individuals without diabetes. Unlike CAT, SOD expressed the clear systematic change with the onset of diabetes. These changes in values of SOD most probably occur due to the faster and more unstable reaction of SOD molecules during the onset of diabetes.

In addition to biochemical changes in glucose levels, the incidence of diabetes caused disorder of dynamics of the relationship between SOD and CAT, through significant changes in their mutual correlations. The system of distribution of CAT and SOD is significantly changed after the metformin therapy. With the onset of diabetes CAT remained normally distributed but SOD lost the allocation system. It is assumed that the breakdown of SOD distribution system is regulatory, due to incomplete exhaustion of CAT and regulatory feedback mechanism of reduced production of hydrogen peroxide by SOD, in order to maintain the normal functioning of CAT. A study by Goth et al. [14], which found that individuals with CAT insufficiency have significantly lower levels of SOD, supports this assumption.

After the treatment period, distribution of both enzymes transforms into uniform. The equilibrium form of uniform distribution without expressed statistical modulepoints out the role of treatment in supporting the balance of these enzymes. In doing so, it does not matter whether values of enzymes are back to the level of the control group or to the level of the group of obese individuals without diabetes, but it is important that the relationship of SOD and CAT returns to the relationship that existed before the onset of diabetes. Correlation of enzymes in the control group, in the group of obese individuals without diabetes, and in the group of T2DM patients after the treatment period was significantly same, and this correlation is significantly different in the group of T2DM patients at the baseline. According to the response to the metformin therapy, it appears that the functional relationship of enzymes is more important than their individual values. This indicates that exhaustion of SOD and CAT with mandatory

disorder in their correlations can predict the development of T2DM. Heuristic search for values of critical exhaustion of the enzyme generated the value of $CAT = -135$ U/mL. T2DM patients at the baseline with CAT less negative than this cut-off point had significantly higher fasting plasma glucose than patients with CAT which was more negative this cut-off value. Setting the cut-off value of SOD at 270 U/g of hemoglobin revealed that within the same group of patients, ones with lower levels of this enzyme do not have significantly higher values of fasting plasma glucose in comparison to patients with greater levels of SOD . This reflects the most stable level of antioxidant capacity and significance of CAT . However, synergistic effect of CAT and SOD is significant, so at the baseline, T2DM patients with lower level of SOD and lower activity of CAT have significantly higher levels of fasting plasma glucose in comparison with patients with higher SOD value and higher CAT activity. Observed synergistic relationship of enzymes is, at least to some extent, described by Lortz and Tiedge [15] who found that optimal protection of pancreatic β -cells from oxidative damage is provided by combined increased expression of both SOD and CAT . The exhaustion of compensatory mechanisms of CAT and/or its glycosylation in the group of patients with newly diagnosed T2DM likely leads to excessive accumulation of hydrogen peroxide and subsequent suppression of the SOD activity through the feedback mechanism. The suppression of SOD activity may lead to further damage of antioxidative enzymes by accumulated superoxide anions (insufficient elimination of these anions by SOD). However, established critical levels of enzymes and their synergy is losing significance after the additional oxidative load (postprandial state). Significantly superimposed levels of glucose are most probably caused by the increase in insufficiency of antioxidant enzymes after the meal. In addition to the recovery of correlation between enzymes and significant decrease in glucose levels, the metformin therapy has eliminated the existence of the critical interval of enzymes.

In our study, recovery of correlations between enzymes after the metformin treatment may be a result of short duration of diabetes, so this hypothesis should be tested in the larger population of T2DM patients and with a longer period of the follow-up.

Bakala et al. [16] were examining mitochondrial extracts from the liver of experimental animals and found that CAT is the most vulnerable to glycosylation of all antioxidant enzymes. High levels of hydrogen peroxide are present in cells with sufficient quantity of Gpx , but with reduced reserve of CAT [17]. These results are in accordance with ours which report association between higher levels of glucose and lower levels of CAT activity, and even more

pronounced association of higher glucose levels with combined presentation of lower levels of SOD and lower activity of CAT. Since catalase appears also as an anticancer target, the elucidation of mechanisms regulating its expression is an important issue [18].

Goth et al. [19] considered that elevated concentrations of hydrogen peroxide, due to reduced activity of CAT, can contribute to the oxidative damage of pancreatic β -cells, thus reducing the secretion of insulin and leading to the onset of diabetes and the increase in carotid intima media thickness [20]. New research by Goth et al. [21] reports low levels of CAT in T2DM patients but not in patients with type 1 diabetes. Mutual cooperation of antioxidative enzymes is also vital for normal functioning of the organism [22]. Alfa-lipoic acid (ALA) an essential co-enzyme for energy production in mitochondria, demonstrates substantial antioxidant properties and an effect on whole-body physiology like inhibition of glycation reactions and prevention of beta-cell destruction [23]. It has been used in several oxidative-stress models such as diabetes, ischemia-reperfusion injury, cataract and neurodegenerative disorders. The current findings suggest that α -lipoic acid is beneficial and thus should be considered for routine administration in patients with diabetes and peripheral neuropathy [24]. Study of Yang et al. [25] demonstrated that metformin might be effective for reducing blood glucose and promoting glucose uptake and utilization and that ALA might be effective for improving insulin sensitivity and activating insulin-signaling pathways. They demonstrated that ALA enhances hepatic insulin sensitivity and prevents the development of NAFLD, furthermore, ALA ameliorates glucose metabolism by modulating the insulin-signaling pathway.

CONCLUSION

Although significant differences in absolute values of CAT between groups were not observed, significant changes in linear correlation of CAT and SOD, in particular in the group of T2DM patients treated with the metformin therapy, are clearly visible. In cases of low CAT activity caused by exhaustion of physiological reserves followed by the accumulation of hydrogen peroxide, which beside its toxic oxidative action, inhibition of the activity of SOD through the feedback mechanism also occurs. Significantly higher fasting plasma glucose levels are found among group with low CAT activity and low SOD level comparing to the complementary group. Taking into account that CAT is the most vulnerable to glycosylation [26] among all antioxidant enzymes and that it has the highest rate of metabolic turnover among all known enzymes due its physiological role in removing molecules of hydrogen peroxide (as

the most stable oxidative compound), it seems that the activity of this enzyme is the key point of maintaining the oxidative-reduction balance in terms of glycemc control. Certainly, it remains the question of the cause and the effect. If the disorder of relationship of enzymes precedes the development of diabetes, then these conclusions could be used in the individual prevention of diabetes. However, in most cases we do not have data on the onset and on duration of diabetes prior to its diagnosis. Longitudinal follow-up of values of these antioxidant enzymes in obese patients prone to diabetes could lead to an answer. Long-term monitoring of dynamics of these enzymes during the diabetes duration may provide more detailed insight into their function, which may not be identical as in the newly diagnosed T2DM patients. It remains an open question whether long-term treatment with metformin maintains correlation relationships of antioxidant enzymes in the same way as it observed after the three-month treatment period. This may be an important issue because the primary therapeutic response is synchronization of the relationship between antioxidant enzymes, and not the impact on their individual absolute values which are influenced by many other factors.

Conflict of interest: None declared.

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Table 1. Parameters and distributions (mean value and standard deviation) for Superoxyd dismutase (SOD), Catalase (CAT), fasting and postprandial 2h glucose (G0H and G2H) in study groups;

Types of distribution: N – normal; U – uniform; lnN – lognormal; X – undefined

Groups of patients	SOD (U/g) (Figure 1)	CAT (U/mL) (Figure 2)	(G0H) mmol/l (Figure 3)	(G2H) mmol/l
Control group	N (303.28; 57.73)	N (-141.24; 47.13)	N (4.54; 0.35)	N (4.952; 0.654)
Obese group	N (247,05; 55.25)	N (-147.35; 37.61)	N (5.06; 0.54)	N (5.528, 0.965).
T2DM group before treatment	X (283.72; 122.35)	N (-140.62; 39.92)	X (9.48; 3.72).	X (12.74; 5.42).
T2DM group after treatment	U (238.82; 35.91)	U (-128.95; 35.91)	lnN (7.42; 2.45)	lnN (9.307, 3.218)

Figure 1. Distribution and verification of Superoxyd dismutase in the control group, group of obese individuals without diabetes, T2DM patients at the baseline and after the treatment period

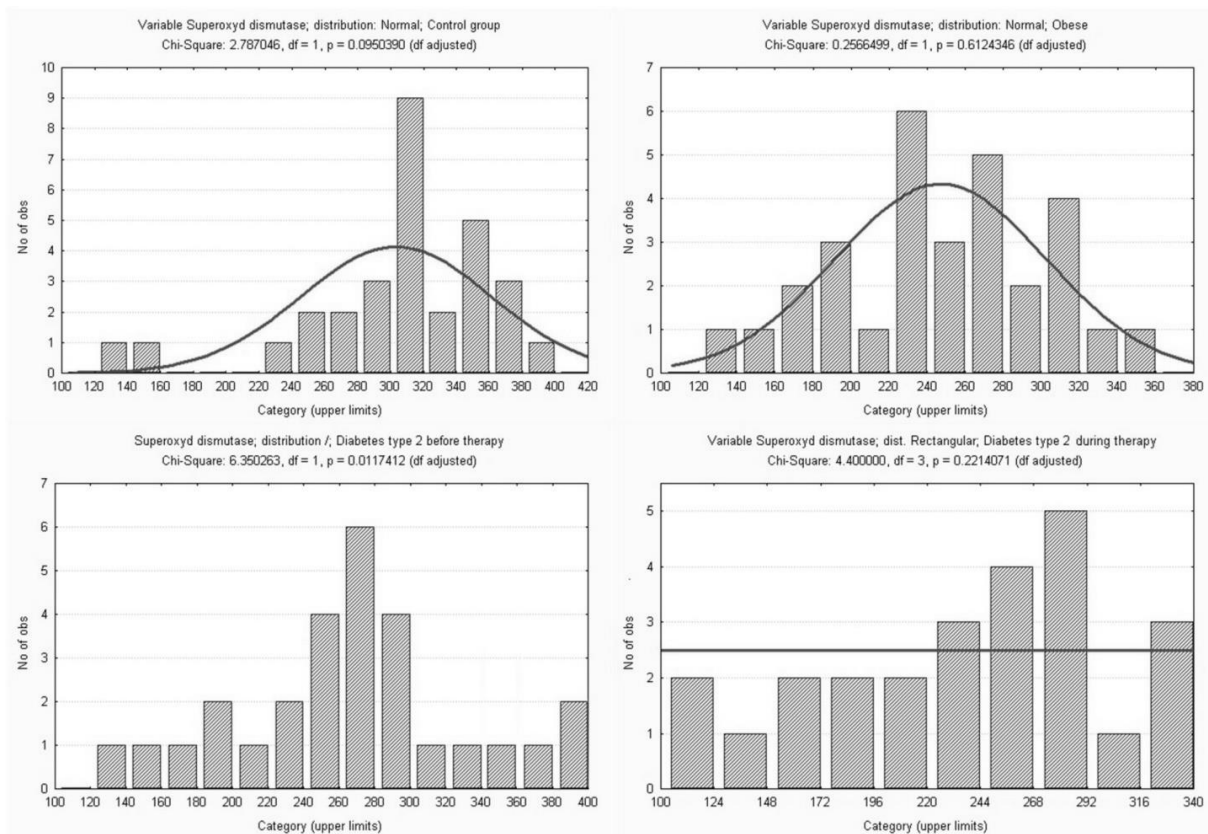
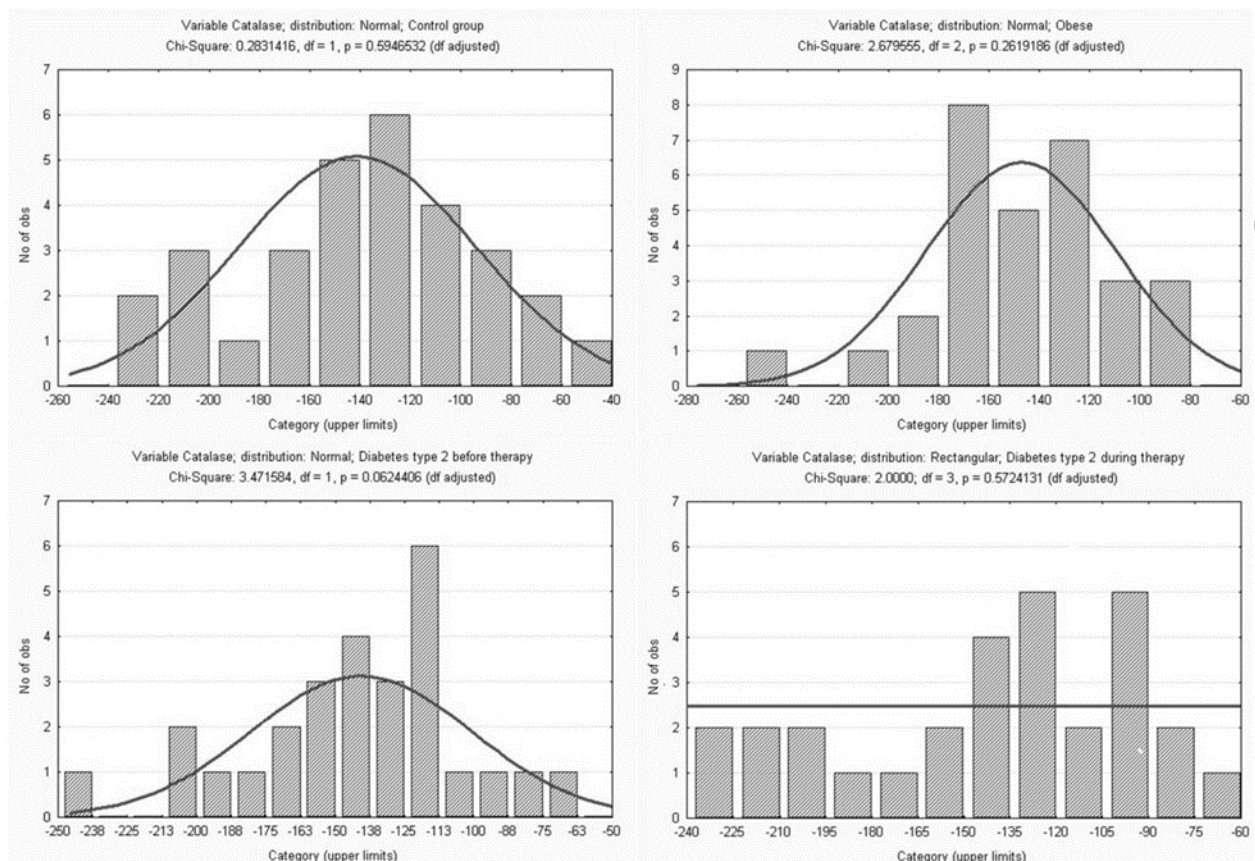
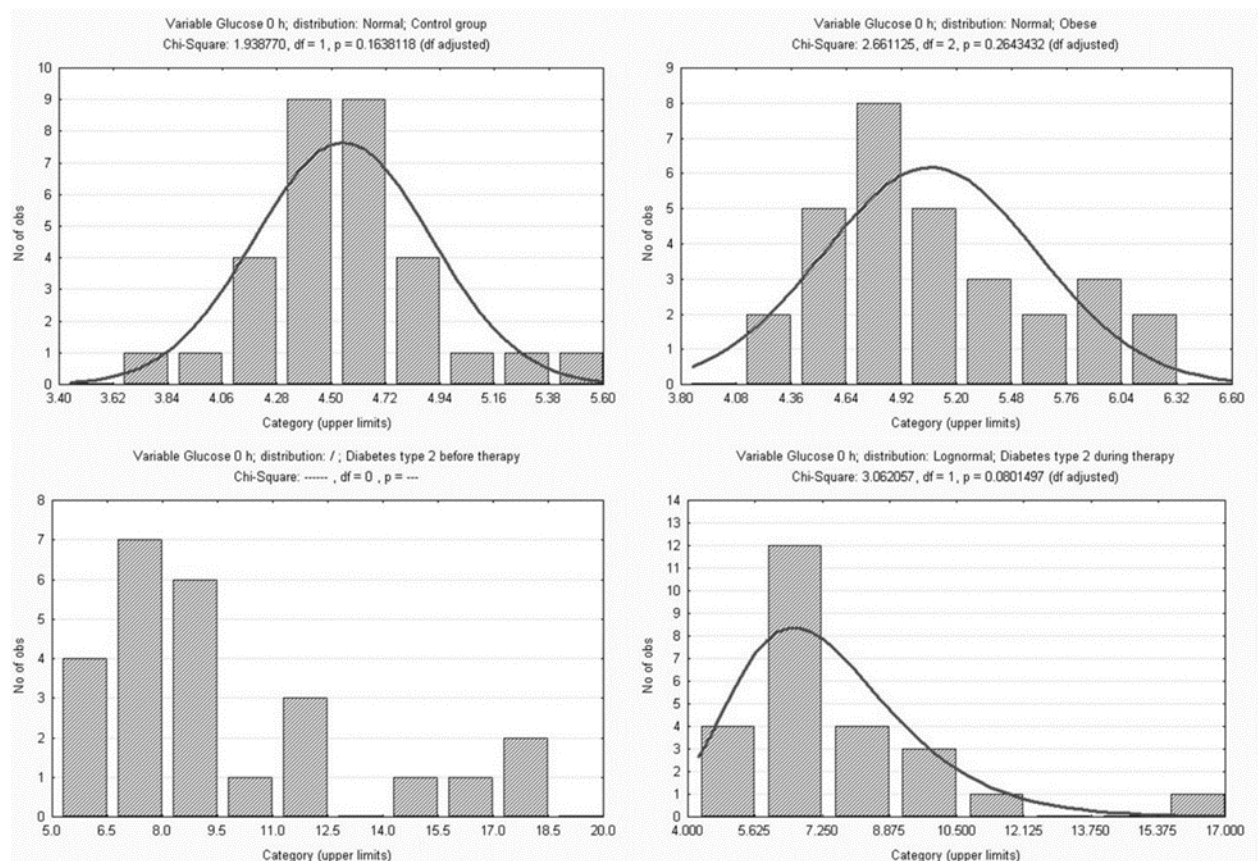


Figure 2. Distribution and verification of Catalase (CAT) in the control group, group of obese individuals without diabetes, T2DM patients at the baseline and after the treatment period



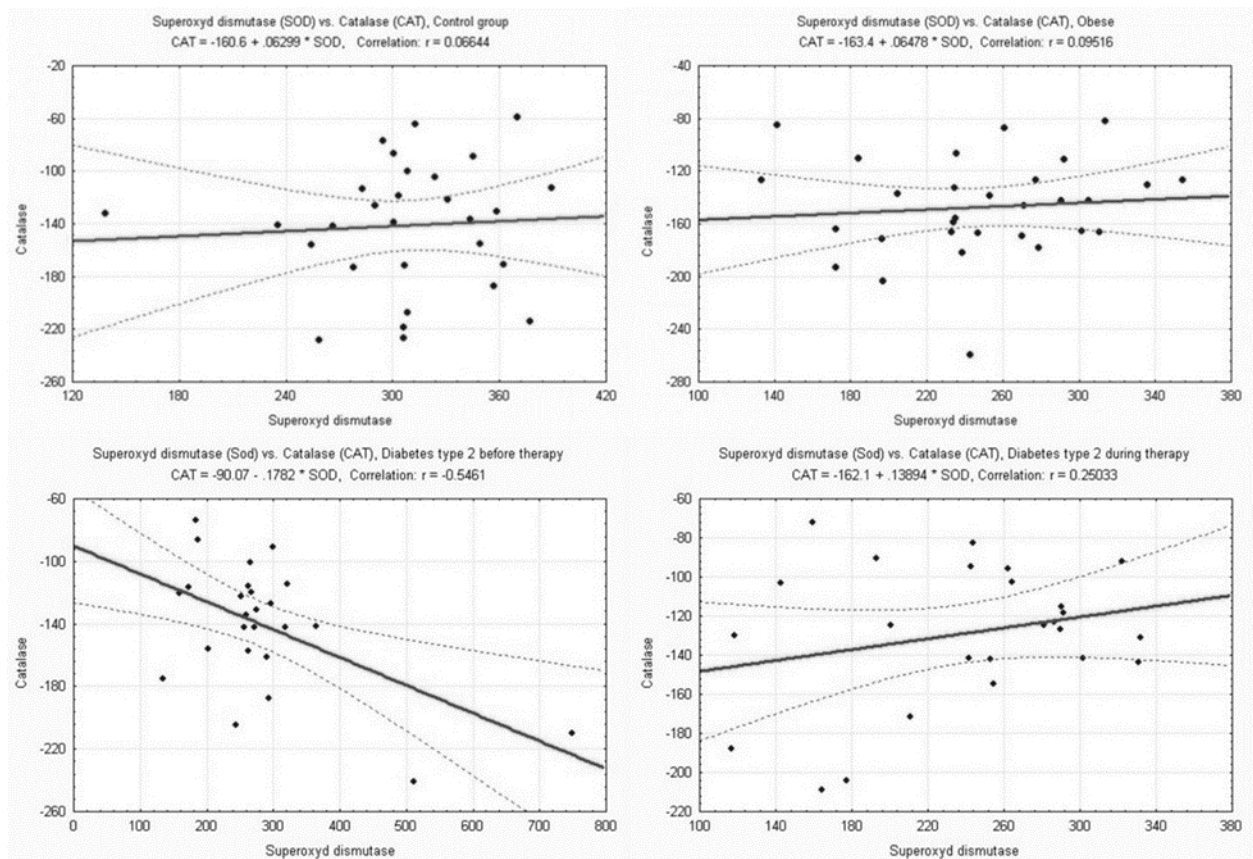
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Figure 3. Distribution and verification of G0H in the control group, group of obese individuals without diabetes, T2DM patients at the baseline and after the treatment period



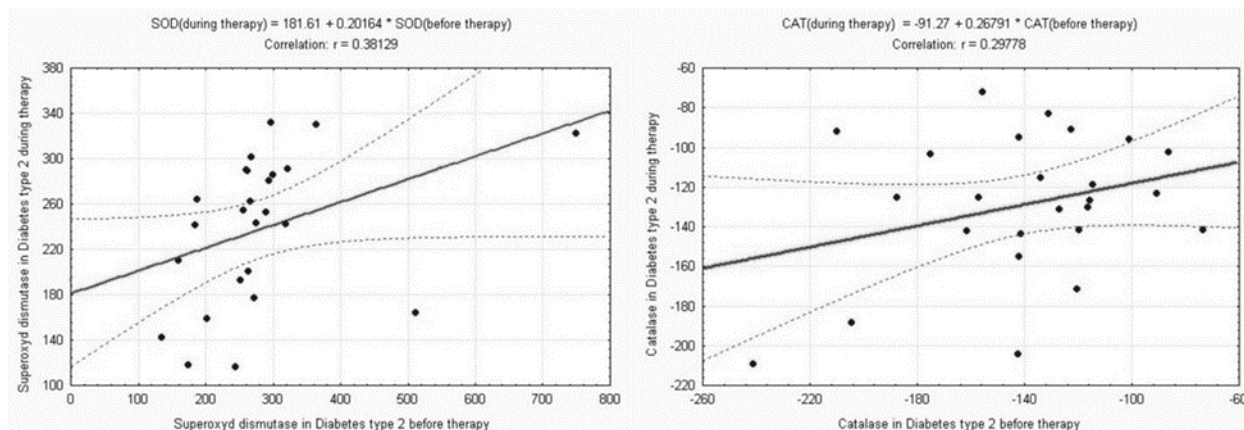
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Figure 4. Equations, correlation coefficients, graphical display of lines of linear regressions of Superoxyd dismutase (SOD) and Catalase (CAT) in control the group, group of obese individuals without diabetes, T2DM patients at the baseline and after the treatment period



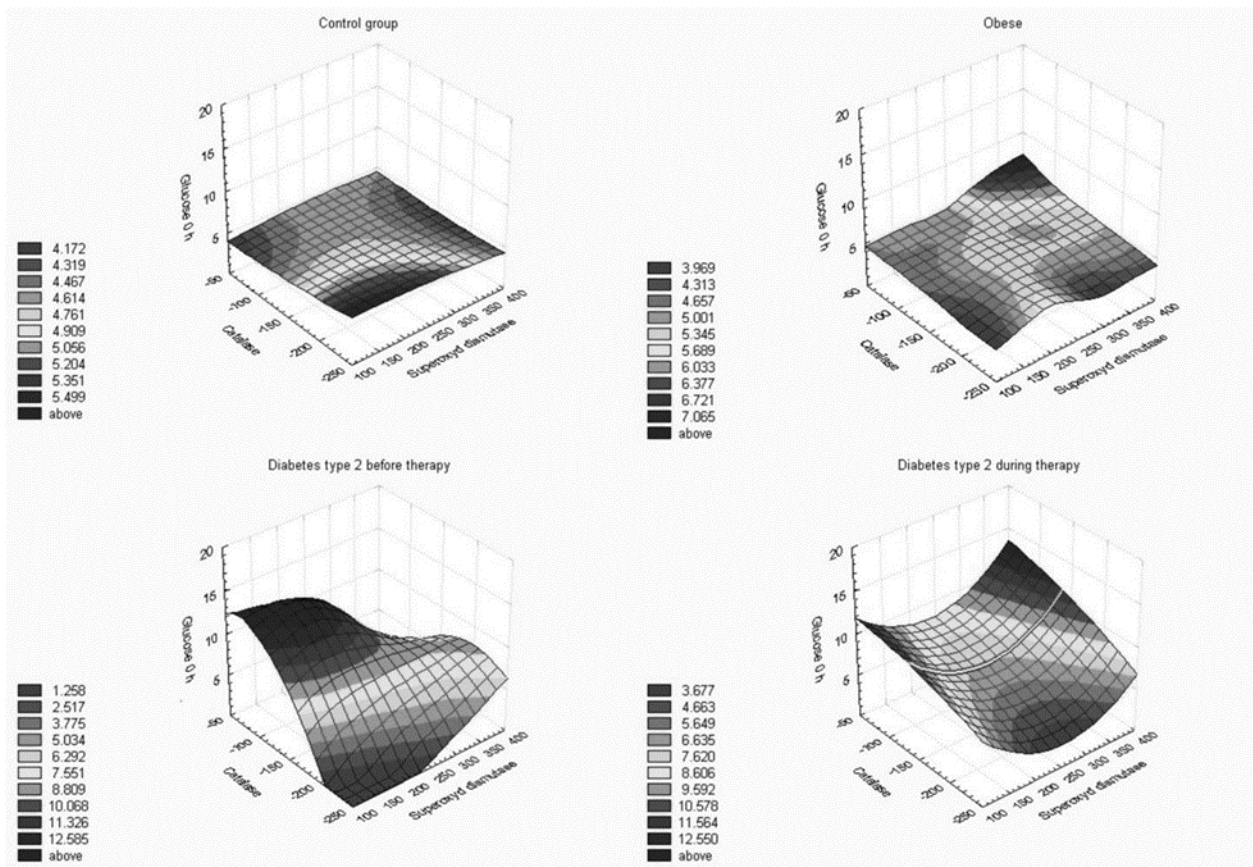
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Figure 5. Effects of metformin therapy on mutual relationships between Superoxyd dismutase (SOD) and Catalase (CAT) values



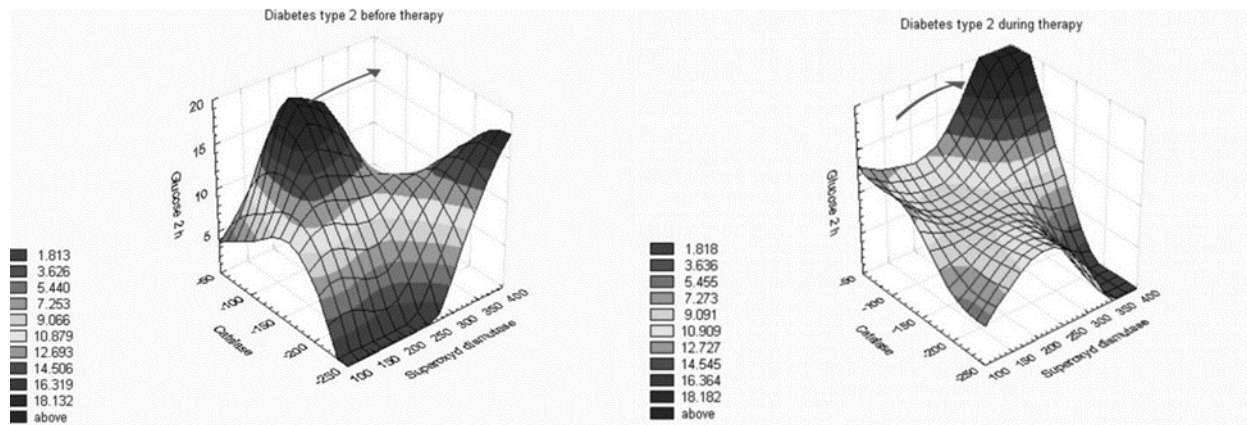
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Figure 6. Two-dimensional G0H as dependent variable in the function of independent variables Superoxyd dismutase (SOD) and Catalase (CAT) in the control group, group of obese individuals without diabetes, T2DM patients at the baseline and after the treatment period



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Figure 7. Two-dimensional G2H as dependent variable in the function of independent variables Superoxyd dismutase (SOD) and Catalase (CAT) in T2DM patients at the baseline and after the treatment period



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