http://dx.doi.org/10.5281/zenodo.4498977

Analysis of peroxidase activity in diabetic retinopathy and in applying various corrective means.

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Abstract

Hyperglycemia stimulates the development of oxidative stress, which in turn is a powerful pathophysiological mechanism for the development of microvascular complications in diabetes. Increased production of reactive oxygen species is observed both during development and during the progression of diabetic retinopathy.

The study was performed on white Wistar rats weighing 180-200 g. According to the tasks, the animals were divided into 7 groups: 1st group - 60 intact animals; Group 2 - 60...
animals in which diabetic retinopathy was simulated without further correction. Group 3 - 60 animals, which simulated diabetic retinopathy with subsequent correction of hyperglycemia; Group 4 - 60 animals in which diabetic retinopathy was simulated with subsequent correction of hyperglycemia, administration of aflibercept and L-arginine solution; Group 5 - 60 animals in which diabetic retinopathy was simulated with subsequent correction of hyperglycemia, administration of aflibercept and bromfenac; Group 6 - 60 animals in which diabetic retinopathy was simulated with subsequent correction of hyperglycemia, administration of aflibercept, L-carnitine and bromfenac; Group 7 - 60 animals, which simulated diabetic retinopathy with subsequent correction of hyperglycemia, the introduction of aflibercept, a solution of L-arginine and citicoline.

The results indicate the development of oxidative stress from the 30th and with subsequent progression on the 60th and 180th days of experimental diabetic retinopathy, which is confirmed by a decrease in peroxidase activity in the 2nd group, the maximum of which is observed in the 3rd stage. Correction with hypoglycemic agents in group 3 had a positive effect, but was not able to restore the activity of the antioxidant enzyme, so there was a need for additional drugs. The use of aflibercept and nitric oxide donor in group 4 to correct the development of diabetic retinopathy had a positive effect on increasing the activity of peroxidase, which peaked on the 180th day of the experiment, but did not reach the control values. The combined administration of bromfenac and aflibercept in group 5 was shown to significantly increase antioxidant activity, but not as significantly as in group 4. Administration of aflibercept, L-carnitine, and bromfenac to group 6 animals was shown to restore antioxidant protection as early as day 30 and was continued on days 60 and 180 of the study, but the results did not reach control values. The combination of metformin, aflibercept, L-arginine and citicoline in rats of the 7th group proved to be the most effective correction, as evidenced by the normalization of peroxidase activity on the 30th and 60th day of the experiment, and on the 180th recovery of marker activity to control values was recorded.

Keywords: experimental diabetic retinopathy; oxidative stress; antioxidants; peroxidase; correction; metformin; aflibercept; L-arginine; citicoline; L-carnitine; bromfenac.

Introduction

Diabetes mellitus (DM) is an urgent problem of the XXI century through the continuous progression of the incidence of both types 1 and 2 of the disease [1]. It should be emphasized that according to the WHO, diabetes mellitus is considered a pandemic of the 21st
In 2017, according to the International Diabetes Federation (IDF), the number of patients with diabetes mellitus is 424.9 million worldwide, which is a worldwide problem for humanity [2]. Macro- and microvascular complications significantly worsen the course of diabetes mellitus and cause the pathogenesis of endothelial dysfunction [2].

Hyperglycemia stimulates the development of oxidative stress, which in turn is a powerful pathophysiological mechanism for the development of microvascular complications in diabetes mellitus [3]. Enhanced production of reactive oxygen species is observed both during the development and progression of diabetic retinopathy. Oxidative stress (OS) underlies the pathogenesis of both insulin resistance and vascular diabetic complications, in particular diabetic retinopathy [4]. With the development of OS, which increases during the toxic effect of hyperglycemia on metabolic processes, an increase in the number of free radicals and inhibition of the antioxidant defense system is observed [5].

It has been proven that oxidative stress not only contributes to the development of retinopathy, but maintains this pathological condition even with normalization of glucose levels [4, 6]. Decreased retinal activity of antioxidant enzymes such as glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase, observed in diabetes, also contribute to the development of oxidative stress [7, 8]. According to one hypothesis, oxidative stress in diabetic retinopathy is a link between metabolic pathways that have been damaged by hyperglycemia. Reactive oxygen species, which are formed in mitochondria, destruct DNA, and thus activate poly-ADP-ribose polymerase [4].

**The aim of the study**: analysis of changes in blood peroxidase activity in experimental diabetic retinopathy and various methods of its correction.

**Materials and methods.** The study was performed on white Wistar rats weighing 180-200 g. According to the tasks, the animals were divided into 7 groups:

- **Group 1** - 60 intact animals;
- **Group 2** - 60 animals in which diabetic retinopathy was simulated without further correction.
- **Group 3** - 60 animals in which diabetic retinopathy was simulated with subsequent correction of hyperglycemia.
- **Group 4** - 60 animals, which simulated diabetic retinopathy with subsequent correction of hyperglycemia, administration of aflibercept and L-arginine solution.
- **Group 5** - 60 animals, which simulated diabetic retinopathy with subsequent correction of hyperglycemia, administration of aflibercept and bromfenac.
Group 6 - 60 animals in which diabetic retinopathy was simulated with subsequent correction of hyperglycemia, administration of aflibercept, L-carnitine and bromfenac.

Group 7 - 60 animals, which simulated diabetic retinopathy with subsequent correction of hyperglycemia, the introduction of aflibercept, a solution of L-arginine and citicoline.

Type 2 diabetes mellitus and diabetic retinopathy were simulated by intraperitoneal administration of streptozotocin (Sigma, USA) dissolved in 0.1 M citrate buffer with a pH of 4.5 [9]. The dose of streptozotocin 55 mg / kg body weight was divided into two injections. The introduction of streptozotocin was preceded by a high-fat diet for 28 days.

*Doses of drugs:*

Hypoglycemic medications - metformin (Merck Sante, manufacture France) - in a dose of 300 mg / kg in drinking form [10] 0.9% sodium chloride solution via syringe with intragastric probe daily.

Administration of L-arginine solution, which is a donor of NO, (SIMESTA, made in China, USP32 quality standard) was carried out by intragastric administration of L-arginine solution in 0.9% sodium chloride solution at a dose of 500 mg / kg [11] through a syringe with intragastric tube. The volume of the solution depended on the weight of the animal and did not exceed 1 ml. The drug was administered once a day before morning feeding, daily for 10 days [11].

Aflibercept (anti-VEGF therapy) was administered as a subconjunctival injection at a dose of 0.08 ml (25 mg / ml) [12].

Bromfenac - instillation of 0.09% solution of eye drops once a day.

L-carnitine (Sigma, USA) was administered in the form of an aqueous solution through a syringe with an intragastric tube at a dose of 25 mg / 100 g of animal weight [13, 14].

Citicoline - 81.8 mg / kg (0.33 ml / kg) was administered intramuscularly once a day.

Withdrawal of animals from the experiment was carried out in three stages:

1<sup>st</sup> stage of the study - the 30<sup>th</sup> day after the start of modeling diabetes mellitus;

2<sup>nd</sup> stage of the study - the 60<sup>th</sup> day after the start of modeling diabetes;

3<sup>rd</sup> stage of research - the 180<sup>th</sup> day after the simulation diabetes.

Animals were removed from the experiment by decapitation under light ether anesthesia in accordance with the "Rules for performing work using experimental animals", approved by the Order of the Ministry of Health of Ukraine № 249 from 01.03.2012 and the Law of Ukraine № 3447-IV "On protection of animals from cruelty" (as amended from 15.12.2009 and from 16.10.2012).
Blood was taken from the retroorbital venous plexus, which lies in orbit behind the eyeball. The puncture was performed in a circular motion with a glass pipette with an extended capillary, the tip of which is ground at an angle of 45°. A conjunctival sac was punctured in the medial corner of the eye between the eyeball and the orbit. After puncture, the pipette was inserted to a depth of 2-4 mm behind the eyeball. Control of entering the venous plexus - filling the capillary of the pipette with blood (Dyakonov AV, Khrikina IS, Hegai AA, etc., 2013).

Statistical processing of the obtained results

To identify changes in the studied parameters (endothelial and inducible NO-synthase activity) between different groups and at different stages, we used parametric statistical methods, which are based on operating with the parameters of statistical distribution (mean and variance).

The methods used are designed for normally distributed data, so we performed a check of all data for normality using the criterion of asymmetry and excess EI Pustylnyk. According to this criterion, the distribution is different from normal if calculated empirical values of skewness and kurtosis do not exceed critical, ie $A_{emp} < A_{cr}$, $E_{emp} < E_{cr}$, where $A_{emp}$ and $E_{emp}$ – calculated values of asymmetry and excess, and

$$A_{cr} = 3 \cdot \sqrt{\frac{6 \cdot (n - 1)}{(n + 1) \cdot (n + 3)}}, \quad E_{cr} = 5 \cdot \frac{24 \cdot n \cdot (n - 2) \cdot (n - 3)}{(n + 2)^2 \cdot (n + 3) \cdot (n + 5)}$$

respectively, their critical values [15].

All the data that we are considering was revealed by normal rosetting, so you can compare the Average values of vibroscopes in pairs. Note that in further comparisons, we perform comparisons in independent samples. These will be comparisons between different groups of animals or a comparison between that there is a group of animals (but since the sample does NOT have correspondences between animals, they will also be Independent).

Before comparing the averages of the two samples, it should be determined whether the variances are homogeneous. For this purpose it is necessary to carry out check for homosketicity (homogeneity of dispersions).

Statistical hypotheses are as follows:

$H_0$: the variance in group 1 does not differ from the variance in group 2.

$H_1$: the variance in group 1 is greater than the variance in group 2.

Hypotheses in the criterion are directed, so the criterion is one-sided. Hypothesis $H_0$ is rejected when $F_{emp} > F_{cr}$. This is evidenced by the p-value - the probability of error to
reject the null hypothesis when it is correct. In various experiments, take \( H_0 \) when \( p\text{-value} > \) (significance level set), and reject \( H_0 \) when \( p\text{-value} < \). In all subsequent calculations, we chose a standard level of significance = 0.05.

The comparison of averages is performed using Student’s t-test. When comparing the average directed hypotheses will be as follows:

\( H_0 \): the average of group 1 does not differ from the average of group 2.

\( H_1 \): the average of group 1 is greater than the average of group 2.

To make a decision, the absolute value of the calculated \( t \) is compared with a one-sided critical. If \( |t_{emp}| < t_{cr} \), then the null hypothesis cannot be rejected. Here it is similarly possible to draw a conclusion and on \( p\text{-value} \).

We will perform all tests in the statistical package PASW Statistics 18. We will use the t-test procedure for independent samples, which immediately compares variances and means.

**The results of the study and their discussion:**

A necessary link in antioxidant protection is a group of enzymes that neutralize hydrogen peroxide. One such enzyme is peroxidase [16]. This enzyme is able to catalyze oxidase and peroxidase oxidation and thus is able to take an active part in many chemical reactions of the body [17]. Peroxidase is able to control the level of hydrogen peroxide by reducing it to water [16]. It is a two-component enzyme class oxoreductase, which consists of hematin and apoenzyme [17]. It belongs to a large group of enzymes that channel the oxidation reactions of inorganic and organic substrates using organic peroxides as electron acceptors or hydrogen peroxide [16]:

\[
\begin{align*}
2\text{HX} + \text{H}_2\text{O}_2 & \rightarrow 2\text{X} + 2\text{H}_2\text{O}; \\
2\text{HX} + \text{ROOH} & \rightarrow 2\text{X} + \text{H}_2\text{O} + \text{ROH},
\end{align*}
\]

where \( \text{HX} \) - restored substrate, \( \text{X} \) - oxidized substrate

Peroxidase is a link in the electron transfer chain in the mitochondrial alternative respiratory chain. It participates in redox reactions of photosynthesis, reduction of nitrates and nitrites of nitrogen metabolism, respiratory processes, participates in the regulation of development and organogenesis [16, 18].

In the cell, peroxidases play an important role in maintaining molecules in a restored state, which is one of the keys to homeostasis. Peroxidases belong to inducible enzymes, which under the influence of various factors can change their lysoforms or increase the activity of
existing molecular forms [19]. The results of the study of peroxidase activity in the conditions of our experiment are presented in Table 1.

Table 1. - The level of peroxidase in the blood of experimental animals with simulated diabetic retinopathy and with different methods of its correction on the 30th, 60th and 180th day (M ± m)

<table>
<thead>
<tr>
<th>Stages</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 group</td>
<td>152,1±2,63</td>
<td>152,1±3,89</td>
<td>152,1±3,02</td>
</tr>
<tr>
<td>2 group</td>
<td>101,89±1,88</td>
<td>92,3±3,43</td>
<td>86,9±3,22</td>
</tr>
<tr>
<td>3 group</td>
<td>104,3±2,92</td>
<td>108,9±2,57</td>
<td>112,1±3,15</td>
</tr>
<tr>
<td>4 group</td>
<td>112,4±3,1</td>
<td>121,7±2,86</td>
<td>124,6±2,43</td>
</tr>
<tr>
<td>5 group</td>
<td>109,89±2,26</td>
<td>114,2±3,08</td>
<td>120,1±2,74</td>
</tr>
<tr>
<td>6 group</td>
<td>124,7±3,08</td>
<td>129,8±2,96</td>
<td>132,3±3,58</td>
</tr>
<tr>
<td>7 group</td>
<td>134,2±2,63</td>
<td>141,9±3,11</td>
<td>150,6±3,05</td>
</tr>
</tbody>
</table>

In the second group, in which the pathological process was not corrected, a decrease in this marker by 49.28% (p <0.001) relative to the norm at the first stage was revealed. At the second stage, the activity of the indicator is 64.78% lower (p <0.001) compared with the data of intact animals and by 10.39% (p <0.01) relative to the results of the first stage. At the third stage, the activity of this marker is 75.03% (p <0.001) lower than in the intact group. Compared with the first stage, there is a decrease of 17.25% (p <0.001).

In group No. 3, it was found that at the first stage, the peroxidase activity was lower by 45.83% (p <0.001) compared with the 1st group. No differences were found in relation to group # 2. At the second stage, the difference from the intact group was 39.67% (p <0.001) towards a decrease in activity, and compared with group No. 2, the activity was 15.24% higher (p <0.001). At the third stage, the enzyme activity slightly increased - by 6.96% (p <0.05) compared with the first stage. It is lower by 35.68% (p <0.001) relative to the intact group, and by 22.48% (p <0.001) higher relative to group No. 2.

In the 4th group, at the first stage, the peroxidase activity is 35.33% lower (p <0.001) compared with group No. 1, and compared with groups No. 2 and No. 3, an improvement of 9.35% is observed (p <0, 01) and 7.2% (p <0.05), respectively. At the second stage, the enzyme activity is 7.64% higher (p <0.05) compared to the previous one. Relative to group No. 1, it is lower by 24.97% (p <0.001), and compared with the results of groups 2 and 3, it is higher by 24.16% (p <0.001) and 10.52% (p <0.001 ) respectively. At the third stage, in comparison with the first, the result is the best by 9.79% (p <0.01). In comparison with the
data of the intact group, the activity of the marker is reduced by 22.07% (p <0.001). In relation to the 2nd group, the activity of the marker is higher by 30.26% (p <0.001), and in comparison with group No. 3 - by 10.03% (p <0.01).

In the fifth group, on the thirtieth day of the experiment, the activity of the marker is 38.41% (p <0.001) lower than in the intact group. In comparison with the 2nd group, the activity is 7.28% higher (p <0.01). No statistically significant differences were found with respect to the 3rd and 4th groups. At the second stage (the sixtyieth day of the study), the marker activity is 33.18% lower (p <0.001). Poriviano with group 2, the activity is less reduced by 19.18% (p <0.001). No statistically significant differences were found with respect to the 3rd group. And in comparison with group No. 4, the peroxidase activity is lower by 6.57% (p <0.05). At the third stage, the activity is 8.5% higher (p <0.01) compared to the first stage. With respect to the intact group, the level of activity is lower by 26.64% (p <0.001), and compared with the 2nd group - higher by 27.64% (p <0.001). Compared with group No. 3, the activity of the marker is higher by 6.66% (p <0.05). No statistically significant differences were found with respect to group 5.

In the sixth group, according to the first stage, the activity of lower peroxidases was 21.98% (p <0.001) compared with group No. 1, and by 18.29% (p <0.001) compared with group No. 2. With respect to all subsequent groups, in which the modeled pathology was corrected, the marker activity in the 6th group was higher: by 16.36% (p <0.001) compared to the third group, by 9.86% (p <0.01) compared from the 4th, and by 11.87% (p <0.001) compared with group No. 5. At the second stage, the value of the indicator was lower by 17.18% (p <0.001) compared with group No. 1. Relative to the 2nd group, the activity is higher by 28.89% (p <0.001), relative to the 3rd - by 16.1% (p <0.001), comparable to the 4th and 5th groups, the activity was also not painful - by 6.24% (p <0.05) and 12.02% (p <0.001), respectively. At the third stage, the activity of the marker is 14.96% (p <0.001) of the lower relative to the intact group. Compared with the 2nd group, the activity is 34.32% higher (p <0.001), compared with the third - by 15.27% (p <0.001), compared with the 4th - more by 5.82% (p <0.05), and in relation to the 5th - by 9.22% (p <0.01).

In group No. 7, at the first stage, the activity of the lower one by 13.34% (p <0.001) relative to the intact group of animals. The threshold value with the 2nd group of increased activity activity by 24.07% (p <0.001), the threshold value with the third group - by 22.28% (p <0.001), in the threshold value with the 4th group - by 16.25% (p <0.001). Relative to the 5th group, the activity is the best by 18.11% (p <0.001), and in relation to the 6th - by 7.08% (p <0.05). At the second level, the identification of the marker activity by 5.43% (p <0.05) is
equal to the 30th day. Relative to the intact group, the activity is lower by 7.18% (p <0.05), but with group No. 2 it is higher by 34.95% (p <0.001). There is a positive trend in comparison with all groups in which the pathological process was corrected: the peroxidase activity is higher by 23.26% (p <0.001) compared to the third group, by 14.24% (p <0.001) compared to the fourth, by 19.52% (p <0.001) in the defect with group No. 5 and by 8.53% (p <0.01) in the defect with the sixth. At the third stage of the study, the normal activity of the study of the indicator was found: by 10.89% (p <0.001) equally with the indicated value 5.77% (p <0.05) was generated from the second, collected groups created earlier (which certainly indicates efficiency of correction). Peroxidase activity with group No. 2 is higher by 42.30% (p <0.001). Also, a more pronounced positive effect was found in porriovanni in all preliminary groups in which DR was carried out: by 25.56% (p <0.001) relative to the third group, by 17.26% (p <0.001) - relative to the fourth by 20.25% (p <0.001) equally with the fifth group and 12.15% (p <0.001) equally with the sixth.

We can state the effectiveness of the application of the correction method in the 6th group for the normalization of peroxidase activity, although the effectiveness is not one hundred percent. In group No. 7, more pronounced positive results were found - the activity of the indicator increased to normal values.

The results of studying the peroxidase activity under experimental conditions are clearly illustrated in Fig. 1.

(A) Box-plots illustrate the distribution of peroxidase activity values in each group at the first stage of the study (n = 20 in each of the study groups). (B) Box-rafts illustrate the distribution of peroxidase activity values in each group at the second stage of the study (n = 20 in each of the study groups). (B) Box-plots illustrate the distribution of peroxidase activity values in each group at the third stage of the study (n = 20 in each of the study groups)
Conclusions:
1. The results indicate the development of oxidative stress, starting from the 30th and with subsequent progression on the 60th and 180th days of experimental diabetic retinopathy, which is confirmed by a decrease in peroxidase activity in the 2nd group, the maximum of which is observed at 3-th stage.

2. Correction with hypoglycemic agents in group 3 had a positive effect, but was not able to restore the activity of the antioxidant enzyme, so there was a need for additional drugs.

3. The use of aflibercept and nitric oxide donor in group 4 to correct the development of diabetic retinopathy had a positive effect on increasing the activity of peroxidase, the maximum of which occurred on the 180th day of the experiment, but did not reach the control values.

4. It was proved that the combined administration of bromfenac and aflibercept in group 5 significantly increased the antioxidant activity, but not as significantly as in group 4.
5. It was proved that the administration of aflibercept, L-carnitine and bromfenac to animals of the 6th group restored antioxidant protection on the 30th and was continued on the 60th and 180th day of the study, but the results did not reach the control values.

6. The most effective correction was a combination of metformin, aflibercept, L-arginine and citicoline in rats of group 7, as evidenced by the normalization of peroxidase activity on the 30th and 60th day of the experiment, and on the 180th was recorded restoration of marker activity to control indicators.

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