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The Effect of Ecdystene on the Activity of Matrix Metalloproteinases in Experimental Alloxan Diabetes in Rats

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Abstract
The article presents data on the study of the influence of "Ecdystene" on the development of alloxane diabetes, the activity of matrix metalloproteinases, and the content level of their inhibitors. Metalloproteinases are involved in various physiological and pathological processes requiring proliferation and migration of cells and, consequently, restructuring of the extracellular matrix. When applied for treatment during 14 and 21 days, Ecdystene surpasses glucose and Retabolil in terms of hypoglycemic action. In alloxane diabetes, there is a reliable increase in the activity of metalloproteinases, especially on the 14th day of its development. Contrary to comparison preparations, Ecdystene has a more pronounced effect on metalloproteinase activity. Treatment with Ecdystene for 14 and 21 days contributed to an increase in the TIMP-1 content by 2.2 and 1.5 times compared to the untreated group. Glucose and Retabolil increased the content of TIMP-1 on the 7th and 14th days of treatment by 1.26; 0.9 and 1.48; 1.17 times compared to the untreated group. Changes in the activity of MMPs contribute to a decrease in catabolism of the components of the extracellular matrix. To achieve high efficiency in the treatment of diabetes in the clinic, it is recommended to use Ecdystene in combination with other drugs.

Keywords Diabetes mellitus · Matrix metalloproteinases · Metalloproteinase inhibitors · Alloxane · Rats · Ecdystene

1 Introduction

The prevalence of diabetes in human populations is 1–8.6% on average; among them, the incidence in children and adolescents is approximately 0.1–0.3%. Given the undiagnosed forms, in some countries, this number can reach 6%. According to the International Diabetes Federation, as of January 1, 2016, about 415 million people aged 20 to 79 years in the world are suffering from diabetes, and half of them do not know about their disease [1].

Today, two-thirds of all people with diabetes mellitus (DM) live in developed countries, but in developing countries, the incidence rate is especially high. Thus, diabetes spreads rapidly, affecting more and more people. By 2025, the prevalence of this disease in economically developed countries will be 7.6%, and in developing countries, it will reach 4.9% [2].

Diabetes is a condition where patients have trouble absorbing, using, and storing glucose. It is a progressive condition which can entail serious complications in the long term, such as coronary thrombosis, vision problems, blindness, stroke, neuropathy, amputation, and kidney disease.

In a healthy person, blood sugar levels can increase slightly and then go back to normal as the body converts the glucose into energy or stores it for later. In a diabetic, the system does not work, either because it has been overloaded (Type 2 diabetes) or, more rarely, due to a hereditary deficiency (Type 1 diabetes) [3, 4].

One of the problems with diabetes is cell matrix remodeling. MMPs play a role in this process. Determination of the content of MMPs and their inhibitors in blood plasma is a new approach to assessing the processes of remodeling of the extracellular matrix in diabetes [5]. The elimination of hyperglycemia, apparently, normalizes matrix catabolism in diabetes. Insulin has been shown to increase the ability

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of mesangiocytes (deep cells) to break down extracellular matrix components, probably due to the activation of MMP-2. Insulin therapy prevents an increase in TIMP-1 expression and a decrease in matrix degradation in experimental diabetes in rats.

More than 30 MMPs have been discovered to date; they are divided into 6 different groups. They differ in molecular structure, substrate specificity, and tissue distribution. Of those groups, the MMP-2 and MMP-9 types are the most studied. These enzymes play a leading role in the breakdown of type IV collagen and other components of the matrix in the kidneys. MMPs from the group of collagenases, stromelysins, and matrilysins also participate in the breakdown of matrix components and provide proteolysis in the pericellular space.

Matrix metalloproteinases belong to the family of zinc metalloproteinases, the function of which is associated with the exchange of proteins of the intercellular matrix. These enzymes play a decisive role in the development of physiological processes such as tissue morphogenesis, resorption, and remodeling, cell migration, adhesion, differentiation, and proliferation, as well as in pathological conditions (rheumatoid arthritis, glomerulonephritis, periodontitis, corneal ulceration, etc.) [6]. MMPs play an essential role in the generalization of tumor invasion and metastasis.

MMPs are synthesized and secreted by several types of cells: fibroblasts, epithelial cells, phagocytes, lymphocytes, and tumor cells.

More than 20 enzymes in the MMP family have been described so far. Based on the primary structure, substrate specificity, and cell localization, they are usually divided into 5 main subfamilies: collagenases, gelatinases, stromelysins, matrilines, and membrane-bound MMPs (MB-MMP). However, some recently described and insufficiently studied MMPs do not belong to any of the named subfamilies and they are separated into the "other enzymes" group. Also, 4 representatives of the family of tissue inhibitors of MMP (TIMP) have been discovered [7].

All MMPs are characterized by the presence of Zn^{2+} ions in the active center and the need for Ca^{2+} ions to stabilize the molecule. The molecules of almost all MMPs contain several different domains, each of which is responsible for a specific function: latency sustention, secretion, substrate specificity, and catalysis. Besides, all MMPs carry a common conserved sequence. A prodomain containing a conserved sequence is necessary to preserve MMP in latent form and is cleaved during proenzyme activation. The catalytic domain includes three conserved histidine residues in complex with Zn^{2+} ions. The C-terminated part of the molecule includes a hemopexin-like domain, which is responsible for substrate specificity and interaction with cell surface receptors. All MMPs are synthesized as a proenzyme (pro-MMP) and can be secreted in a latent form. Proenzyme activation occurs with the participation of a number of proteases outside the cell or on its surface [8].

The activation of MMP on the cell surface is important for the degradation of the matrix in the pericellular space, which is necessary for cell migration and proliferation. It is necessary to emphasize the special role of MB-MMP in connection with the fact that these proteinases not only are involved in the degradation of the matrix at the site of their localization on the cell surface, but can also trigger the cascade of proteolytic reactions catalyzed by MMP by activating collagenase-3 and gelatinase A, initiating and enhancing the decay of the matrix [9].

A necessary condition for the normal course of physiological processes in the intercellular matrix is to maintain a balance between the activity of MMPs and their inhibitors. Violation of this balance can have a profound effect on the composition of the intercellular matrix and affect various cell functions, including adhesion, migration, and differentiation [2].

A decrease in MMP activity or increased synthesis of TIMP inhibitors helps to reduce the metabolism of extracellular matrix components and constitutes a biochemical basis for matrix degradation. An increase in blood sugar plays a leading role in the development of metabolic disorders of the matrix in diabetes.

Modern studies on the molecular-cellular mechanisms of action of various drugs on the development of diabetes mellitus (DM) have shown that the multifaceted nature of the chosen problem is determined by the many drugs used in the treatment of diabetes and their insufficient effectiveness associated with the complex pathogenesis of this disease [10]. This is why few studies have been conducted to investigate the pathogenetic and molecular mechanisms of action of domestic drugs, in particular, based on phytoecdysteroids. As a result of many years of research, the Institute of Plant Chemistry of the Academy of Sciences of the Republic of Uzbekistan has developed a drug called Ecdysten for the correction of metabolic processes. Ecdysten (also called ecdistenum, ecdysterone, ectysterone, 20 Beta-Hydroxyecdysterone, turkesterone, ponasterone, ecdysone, ecdystene) is a natural steroid compound isolated from the roots and rhizomes of the maral root (Rhaponticum carthamoides). In 1998, the drug was registered with the General Directorate for Quality Control of Medicines and Medical Equipment under the Ministry of Health of the Republic of Uzbekistan under number 87/848/2.

The study showed that Ecdysten is low toxic [11] and has a wide range of biological effects. When introduced into animals, a pronounced tonic and restorative effect is observed [12]. It increases the adaptive capabilities of the animal organism concerning stressing environmental factors and improves their dynamic performance. An essential point in Ecdysten's mechanism of action is its ability to activate

protein biosynthesis in various organs and tissues similar to the known steroid anabolic drugs (nerobol, retabolil (nandrolone decanoate)) [13].

Thus, the purpose of research was to study the effects of Ecdysten on the activity of matrix metalloproteinases in experimental alloxan diabetes in rats.

2 Materials and methods

In the course of the research, 4 series of experiments were carried out on 130 outbred male rats weighing 120–130 g fed with a standard diet. The experiments were guided by the "European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes" (Strasbourg, 1985). In the first series of experiments, the content of metalloproteinases (30 rats) in alloxan diabetes (AD) was studied. AD was caused by a single intraperitoneal injection of alloxan (OOO Yugsintez, Ukraine). Alloxan was dissolved in phosphate buffer (pH 7.4) and was administered at one dose delivery of 13 mg per 100 g of body weight [14]. Fourteen and 21 days after the administration of alloxan, after the complete elimination of the toxic effect of alloxan from the body, animals were decapitated at the same time and studies of blood serum were performed.

In the second series of experiments on 30 rats, the influence of Ecdysten on the development of blood pressure was studied. Starting from the 7th day of the development of blood pressure for 14 and 21 days, the experimental animals were treated with Ecdysten at a dose of 0.143 mg per 100 g of body weight. In animals of the 3rd and 4th series of the experiment (60 rats), the effects of Glucophage (4.28 mg per 100 g of body weight) and Retabolil (0.0714 mg per 100 g of body weight) on the development of AD were studied. The intact group was made of 10 rats.

Glucophage is almost the only known antidiabetic agent that reduces the risk of death from diabetes and its serious complications. It reduces the rate of absorption of carbohydrates in the small intestine, increases the sensitivity of peripheral tissues to insulin, inhibits the processes of gluconeogenesis and glycogenolysis in the liver, and reduces systemic hyperinsulinemia.

Retabolil is a synthetic derivative of testosterone, an anabolic drug with prolonged action (reservoired drug). It stimulates protein synthesis in the body, causing retention of nitrogen, calcium, sodium, potassium, chloride, and phosphorus, which leads to an increase in muscle mass and accelerated bone growth, as well as water retention in the body. It has low androgenic activity.

We measured the blood serum levels of matrix metalloproteinases of the family MMP-2, MMP-9, and the blood serum levels of their inhibitor TIMP-1 by enzyme-linked immunosorbent assay (ELISA) using standard test systems from Quantihine, R@DSystems, USA. This test is based on a quantitative sandwich enzyme-linked immunosorbent assay. The optical density was measured using a computer immuno-enzyme analyzer AT-858 (LTD, China) at a wavelength of 450 nm.

The contents of the studied parameters in lysates were measured in nanograms per 1 mg of total protein (ng/mg protein), which was determined by the method of O.H. Lowry et al. The construction of the calibration graph and calculation of the concentrations of the studied parameters MMP-2, MMP-9, and TIMP-1 was carried out according to the levels of linear regression in logarithmic coordinates.

To determine the degree of diabetes development, blood glucose was measured by the glucose-oxidizing method using a portable glucometer manufactured by Satelit (Russia) and a standard set of reagents.

Digital data were statistically processed on a personal computer using a software package for statistical analysis. The arithmetic mean (M) and student criterion (t) were calculated with the account for the error rate (P).

3 Results and discussion

After the introduction of alloxan, blood glucose was measured to verify the presence of AD in experimental animals (Table 1). The table also shows the results of treatment with the studied drugs.

As can be seen from the data in Table 1, the glucose content in the blood of intact rats was 4.85 ± 0.11 mmol/L. During the follow-up control of the alloxan diabetes, we found a significant increase in glucose on the 7th, 14th, and 21st days of its development by 1.7, 1.8, and 1.8 times, respectively. Treatment with Ecdysten, Glucophage, and Retabolil for 7 days did not cause significant changes in glucose compared to untreated animals. Treatment with Ecdysten for 14 days led to a decrease in blood glucose content by 22.8%. At the same time, treatment with Glucophage and Retabolil showed a weak decrease in blood glucose levels by 9.5 and 3.5%, respectively. On the 21st day of Ecdysten treatment, the blood glucose level decreased 1.5 times compared with the untreated group. Meanwhile, Glucophage and Retabolil treatment reduced glucose by 21.6 and 10.2%, respectively.

Thus, Ecdysten is superior to Glucophage and Retabolil in hypoglycemic action, especially during treatment for 14 and 21 days.

Being the key enzymes of the metabolism of the components of connective tissue, MMPs are involved in various physiological and pathological processes that require proliferation and migration of cells and, consequently, the reconstruction of the extracellular matrix. The results of the study of the activity of MMP-2 and MMP-9 are shown in Table 2.

Table 1 Changes in glucose and rat weights in the dynamics of development alloxan diabetes and correction with Ecdysten

№	Group of animals	Blood glucose (mmol/l)	Rat weight (g)
1	Intact	4.85 ± 0.11	144.38 ± 1.071
2	Alloxan diabetes: Day 7	8.37 ± 0.41	140.69 ± 1.053*
	Day 14	8.71 ± 0.37	131.85 ± 1.47
	Day 21	8.95 ± 0.36	138.31 ± 2.47
3	Treatment with Ecdysten: Day 7	8.59 ± 0.32	140.46 ± 3.12
	Day 14	6.78 ± 0.26	139.61 ± 3.75*
	Day 21	5.75 ± 0.24	140.15 ± 3.30*
4	Treatment with Glucophage: Day 7	8.06 ± 0.38	139.71 ± 5.51*
	Day 14	7.88 ± 0.35	140.86 ± 5.92*
	Day 21	7.02 ± 0.33	141.00 ± 5.67*
5	Treatment with Retabolil: Day 7	9.00 ± 0.40	141.54 ± 3.62*
	Day 14	8.41 ± 0.40	144.54 ± 3.14*
	Day 21	8.04 ± 0.38	143.61 ± 4.09*

*$P > 0.05$, in other cases, $P < 0.05$ relative to the intact group

As can be seen from the data in Table 2, the drugs were introduced after 7 days of AD development, and, compared with the intact group, the activity of MMP-2 increases on the 14th and 21st days of AD development, accounting for 36.9 and 11.9%, respectively. Compared to the untreated group, treatment with Ecdysten for 7 and 14 days reduced the activity of MMP-2 by 19.6 and 10.9%, respectively. At the same time, compared to the AD group, Glucophage and Retabolil in the same study period reduced the activity of MMP-2 by 14.6%; 7.9% and 19.9%; 5.8%, respectively.

As of MMP-9, compared with the intact group, there was an increase in its activity on the 14th and 21st days of the experiment by 38.9 and 19.9%, respectively. Correction of the MMP-9 activity with Ecdysten, Glucophage, and Retabolil showed that Ecdysten has the most significant effect on the activity of MMP-9, since after the treatment with Ecdysten, the MMP-9 activity decreased by 30.6 and 11.2% compared with the untreated group on the days 14 and 21, respectively.

Thus, there is a significant increase in the activity of metalloproteinases in AD, especially on day 14 of its development. Among the three drugs studied, Ecdysten has the most significant effect on the activity of metalloproteinases.

The study of the blood serum TIMP-1 showed that, compared with intact animals, in Ecdysten treatment, TIMP-1 levels decrease by 2.06 and 1.35 times on the 14th and 21st days of the development of AD, respectively (Table 3).

Treatment with Ecdysten for 14 and 21 days contributed to an increase in the content of TIMP-1 by 2.2 and 1.5 times compared to the untreated group. On the 7th and 14th days of treatment, Glucophage and Retabolil increased the content of TIMP-1 by 1.26; 0.9 and 1.48; 1.17 times compared with the untreated group (Fig. 1).

4 Conclusion

Thus, compared with Glucophage and Retabolil, Ecdysten causes a more significant decrease in the activity of MMP and increase in the blood serum of TIMP-1 levels in experimental AD in rats. Changing the activity of MMP helps to reduce the catabolism of the extracellular matrix components. Hyperglycemia plays a leading role in the development of matrix catabolism disorders in diabetes. Further study of changes in the activity of MMP and their inhibitors shows promise of developing new methods for the diagnosis and treatment of

Table 3 The effects of Ecdysten on the blood serum TIMP-1 levels during the follow-up control of AD

Groups	TIMP (pg/ml)	
	Day 14	Day 21
Intact group, $n = 6$	1.033 ± 0.027	
AD + H_2O, $n = 7$	0.5 ± 0.06	0.76 ± 0.106
AD + Ecdysten, $n = 7$	1.1 ± 0.04*	1.18 ± 0.073*
AD + Glucophage, $n = 7$	0.63 ± 0.015	0.75 ± 0.011
AD + Retabolil, $n = 7$	0.74 ± 0.004	0.89 ± 0.012

*$P > 0.05$ compared to the intact group

Table 2 The effect of Ecdysten and comparison drugs on the activity of MMP-2 and 9 (ng/ml) in the blood serum of the experimental rats with AD

Groups	MMP-2 activity		MMP-9 activity	
	Day 14	Day 21	Day 14	Day 21
Intact, $n = 6$	4.36 ± 0.02	4.36 ± 0.03	4.31 ± 0.001	4.31 ± 0.002
AD + H_2O, $n = 7$	5.97 ± 0.60[a]	4.88 ± 0.41[a]	5.99 ± 0.026[a]	5.17 ± 0.076[a]
AD + Ecdysten, $n = 7$	4.80 ± 0.29[a,b]	4.35 ± 0.11[a,b]	4.16 ± 0.005[a,b]	4.59 ± 0.041[a]
AD + Glucophage, $n = 7$	5.1 ± 0.21[a,b]	3.91 ± 0.15[a,b]	4.8 ± 0.03[a,b]	4.241 ± 0.026
AD + Retabolil, $n = 7$	5.5 ± 0.18[a,b]	4.60 ± 0.64[a,b]	5.14 ± 0.05[a]	5.17 ± 0.02[a]

[a]$P < 0.05$ relative to the intact group, [b]$P < 0.05$ relative to the control group

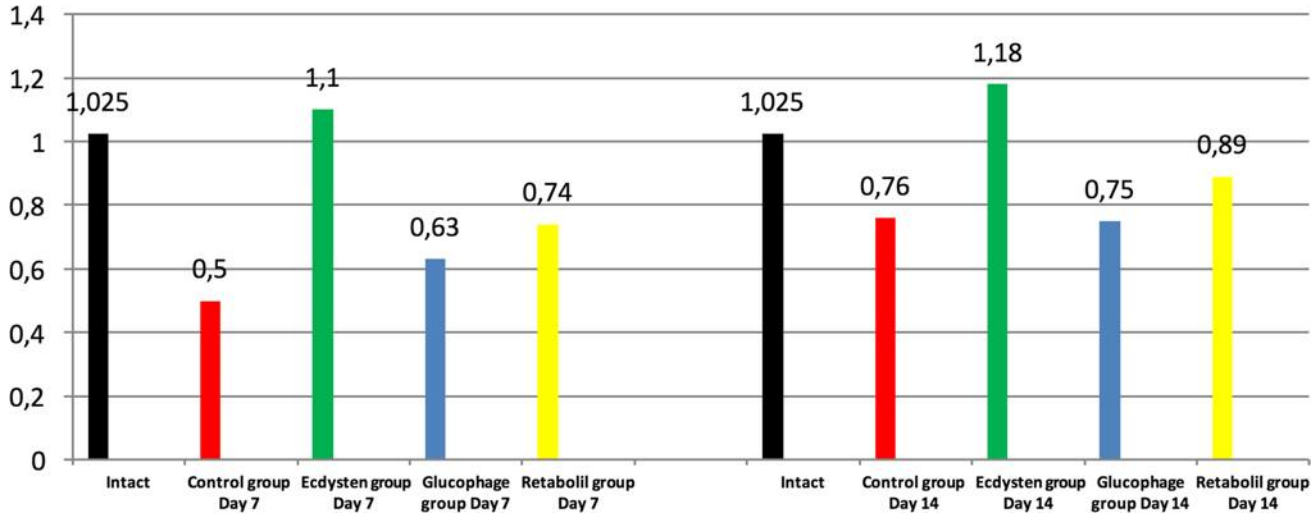

Fig. 1 The effects of Ecdysten on blood serum TIMP-1 levels during the follow-up control of AD

diabetes. In alloxan diabetes, there is an increase in the activity of matrix metalloproteinases, MMP-2 and MMP-9, against the background of a decrease in the content of the TIMP-1 tissue inhibitor of metalloproteinases. Treatment with Ecdysten shows a more pronounced decrease in MMP activity and increase in TIMP-1 content. To achieve high clinical efficiency in the treatment of diabetes mellitus, Ecdysten must be used in combination with other drugs.

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