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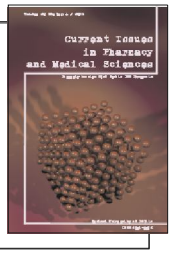

The inhibition of rat leukocytes apoptosis under the condition of experimental diabetes mellitus type 1 by *Galega officinalis L.* extract

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ABSTRACT

The article contains the data of Galega officinalis extract (GOE) nonalkaloid-containing fraction influence on the structural and functional state of leukocytes under the condition of experimental diabetes mellitus type 1 (EDM). Immunohistochemical method has shown the quantitative redistribution of leukocytes containing proapoptotic protein p53 and PARylated [poly(ADP-ribosyl)ated] proteins under EDM conditions and admission of the tested extract. GOE administration to animals with EDM leads to the reduction in the number of cells with positive and strongly positive reaction to the p53 protein and PARylated proteins content, indicating its depressing effect on apoptosis of immune cells.

Keywords: *Galega officinalis L.*, diabetes mellitus, leukocytes, apoptosis, sialic acid

INTRODUCTION

The basis of diabetes development lays in apoptosis mechanisms damage not only of the pancreatic cells, but the immune system cells that are the most important factors of the internal environment stability. The response of immune cells to antigenic stimuli, the nature, dynamics and duration of the immune response, the formation of immunological tolerance is regulated and determined through programmed cell death [8]. More than 150 species of medicinal plants used in treatment of diabetes are mentioned in modern literature. Due to multifunctional physiological effect [7], special attention should be given to Galega officinalis. The evidence provided by the literature proves that prolonged usage of Galega officinalis under the condition of diabetes mellitus leads to the regeneration of Langerhans islets β-cells [13]. This effect may occur due to the inhibition of apoptotic cell death.

The aim of our research work was to study the influence of GOE nonalkaloid fraction on the apoptosis of peripheral blood rats leukocytes under the condition of EDM type 1.

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MATERIALS AND METHODS

The experiments were carried out on white breed less male rats weighting 100–150 g. All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive of 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the Local Ethics Committee. EDM type 1 was caused by inter-abdominal injection of streptozotocin (Sigma, USA) in a dose of 5.5 mg per 100 g body weight of animals.

Two weeks after the induction of EDM, the animals were given GOE nonalkaloid-containing fraction *per os* as an aqueous suspension dose in the quantity of 0.6 g per 1 kg of body weight in 1 ml of volume. The receiving of GOE nonalkaloid-containing fraction was conducted according to the protocol as described earlier [10]. The interest to the investigation of biological effects of investigated nonalkaloid-containing fraction extract was caused by the fact that it has a hypoglycemic effect and is non-toxic [10]. Leukocytes were separated from blood by centrifugation in ficol-triombrast density gradient (ρ= 1,076-1,078 g·cm^{-3}). For detection and visualization of p53 and PARylaited proteins indirect immunoperoxidase method was used. Mouse anti-human antibody to protein

p53 (DAKO, USA, clone DO-7, isotype IgG2b), mouse anti-poly(ADP-ribose) (Trevigen, Inc, USA), antibodies "LSAB®2 Biotinylated Link for Streptavidin HRP/AP ", (DAKO, USA) and avidin-biotin-peroxidase complex "ExtrAvidin Peroxidase "(Sigma, USA), a set of reagents with 3 aminobenzidine "Liquid DAB Substrate Chromogen system "(DAKO, USA) were used in this work. Content analysis of proteins p53 and PARylaited proteins in blood leukocytes was performed by light microscopy. Due to the intensity of dyeing, the studied cells were divided into 3 groups: of negative reaction ($p53^-$ and PARylaited $proteins^-$), positive ($p53^+$ and PARylaited $proteins^+$) and strongly positive ($p53^{++}$ and PARylaited $proteins^{++}$) reaction to the studied proteins. Leukocytes aggregation ability was studied by using two-channel laser aggregation analyzer "LA 230" ("Biola", Russia) in washed leukocytes suspension (2,5 × 106 cells in 1 ml). Lectins: MAA ("Sigma", USA) and SNA ("Sigma", USA) at a concentration of 10 mg/ml were used as aggregation inducers. Statistical analysis of the results was performed using Student's t-test.

RESULTS

EDM development was accompanied by a significant increase of p53: $^+$ (18%) and $p53^{++}$ cells (269%) compared with control, with simultaneous decrease of the number of $p53^-$ cells (21%) (Table 1). These data indicate the intensification of apoptosis and corelate with investigations of other authors concerning the participation of p53 in the pathogenesis of diabetic complications. This way it has been shown on Nod mice that hyperglycemia leads to p53-mediated apoptosis, and in serum of patients with type 1 diabetes anti-p53-autoantibodies are detected and the intensity of p53-induced apoptosis gets increased [6].

The activity of poly(ADP-ribose)polymerase-1 (PARP-1) was estimated by the number PARylated proteins. In the case of diabetes we have found the increase of PARylaited $proteins^+$ and PARylaited $proteins^{++}$ – cells number (61.1% and 101.1%, respectively) and the decrease of PARylaited $proteins^-$ cells by 24.6% compared with control (Table 1). The simultaneous increase in the number of white blood cells containing the high content of p53 and PARylated proteins, confirms the synergism of their action. It has been shown that besides the participation in the process of DNA reparation, PARP-1 transmits a signal range of proteins that carry out coordinated cell response to DNA damage, specifically, leads to rapid accumulation of p53 protein, activation of its binding to DNA. The delay of cell cycle induced by p53 activation, provides the necessary time for DNA repair [11].

The administration of the investigated extract to diabetic animals leads to the reduction of p53 proapoptotic protein content and PARylated proteins in leukocytes, which is proved by the reduction of $p53^+$ and PARylaited $proteins^+$ – cells (23% and 32.8%, respectively), $p53^{++}$ and PARylaited $proteins^+$ – cells (74% and 80.1%, respectively) and increased the amount of $p53^-$ and PARylaited proteins– cells (35% and 32.9%) (Table 1). In the case of GOE administration to healthy animals, we did not observe any significant change in the number of white blood cells that contain p53 protein and PARylated proteins.

The strengthened intensity of immune cells apoptosis leads to changes in the structure of glycocalyx, causes the disruption of their interaction with vascular endothelium and is etiological precondition of the development of diabetic complications and chronic diseases that worsen the condition of patients [12].

For the characteristics of the structures of glycoproteins carbohydrate determinants that form the leukocytes glycocalyx, we have used the following plant lectins: Sambucus nigra agglutinin (SNA), specific to the sequence NeuNAc (α2→6) DGal/DGalNAc and Maackia amurensis agglutinin (MAA), specific to the sequence NeuNAc (α2→3) DGal/DGalNAc.

In the case of EDM the inhibition lectin-induced aggregation of leukocytes has been shown: the maximum degree of SNA- and MAA-induced aggregation decreased by 52.9% and 41.0%, respectively ($p<0.05$). The reducing of the aggregation ability of white blood cells indicates the reduction of receptors and adhesion molecules of glycoprotein nature on the leukocytes surface which contain in their composition both (α2→6) - and (α2→3) - linked sialic acid (Figure. 1). The above-mentioned changes in the profile of surface glycans leukocytes indicate the development of leukocytes apoptosis [12]. GOE

Table 1. Rat peripheral blood leukocytes containing p53 protein and PARylated proteins under conditions of GOE nonalkaloid-containing fraction administration to healthy animals and to animals with EDM (M ± m, n=5–8)

		Control	Control + GOE nonalkaloid-containing fraction	EDM	EDM + GOE nonalkaloid-containing fraction
The number of cells containing protein p53, %	đ53$^-$	56.03±2.98	58.12±1.52	44.35±2.39*	59.79±2.03**
	đ53$^+$	42.50±3.09	40.99±1.60	50.22±2.83*	38.79±2.33**
	đ53^{++}	1.47±0.12	1.89±0.10	5.42±0.36*	1.42±0.22**
The number of cells containing PARylated proteins, %	PARp$^-$	72.96±2.78	76.95±1.24	55.03±2.73*	73.13±1.97**
	PARp$^+$	23.51±2.09	20.99±1.25	37.87±2.10*	25.46±2.15**
	PARp^{++}	3.53±0.35	2.06±0.17	7.10±0.14*	1.41±0.10***

had no effect on the lectininduced aggregation of leukocytes in healthy animals, at the same time predetermined the increase of maximum degree of SNA-induced aggregation (by 45.0%, $p<0.05$) and MAA-induced aggregation (by 57.9%, $p<0.05$) in animals with diabetes.

flow from the cytoplasm to the mitochondria, bypassing the nucleus. In mitochondria, the above mentioned protein undergoes rapid enzymatic deubiquitination, converts into the active form and interacts with the BH4-domain of anti-apoptotic proteins Bcl-XL and Bcl-2 [3].

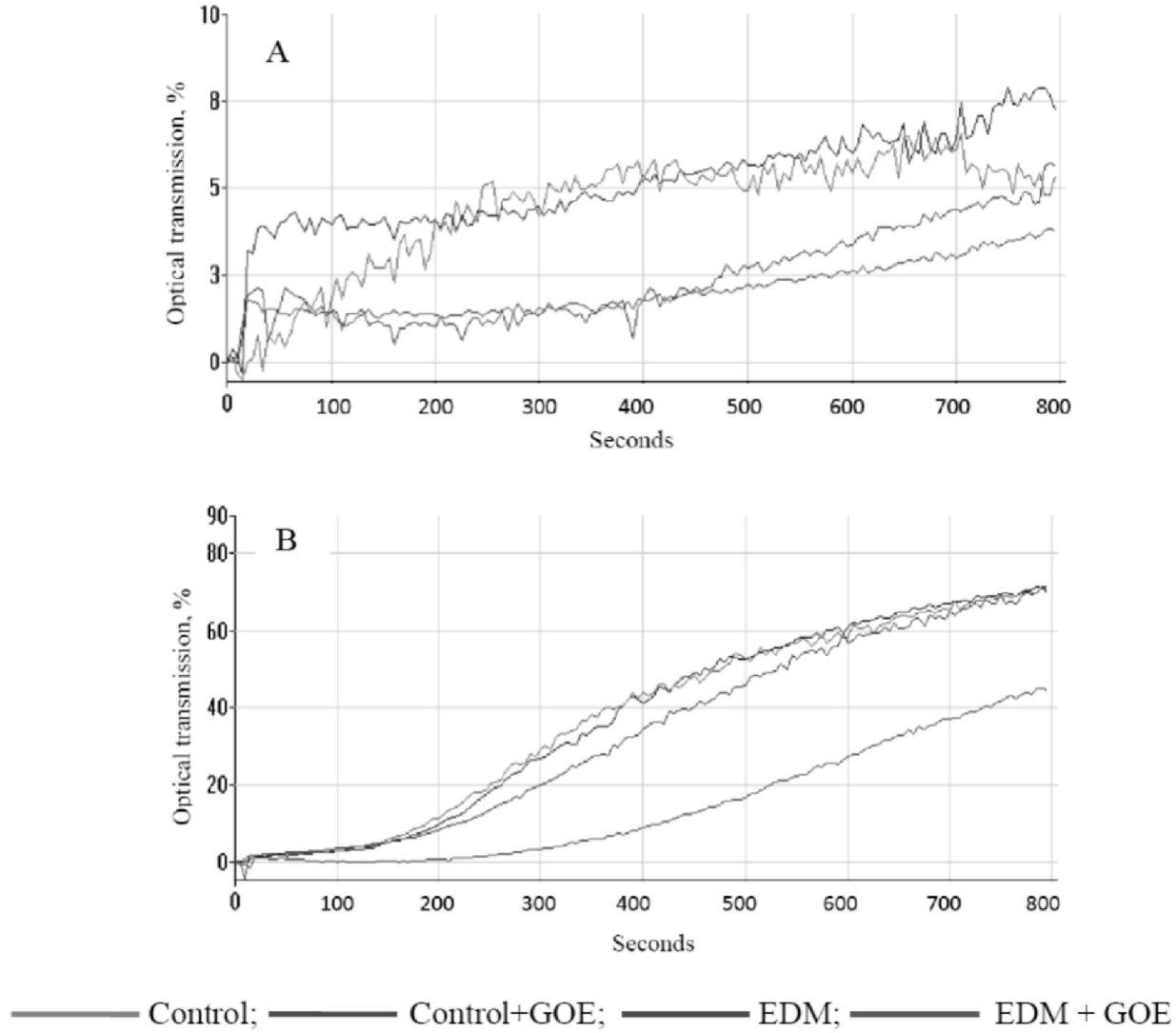

Fig. 1. Typical curves SNA-(A) and MAA-(B)-induced aggregation of leukocytes under conditions of GOE nonalkaloid- containing fraction administration to healthy animals and to animals with EDM

DISCUSSION

The major pathogenetic factor in diabetes is the chronic hyperglycemia, which causes the formation of reactive oxygen species, iNOS activation, NO production with subsequent formation of peroxynitrite and highly reactive hydroxyl radicals which, in turn, cause extensive DNA damage in target cells.

The growth of pro-apoptotic p53 protein content under diabetes conditions occurs in response to DNA damage induced by the function of active oxygen and nitrogen metabolites. Protein p53 cannot only activate genes involved in the development of apoptosis, but can also directly participate in the induction of mitochondrial path of programmed cell death. After the activation, p53 is able to

Binding with antiapoptotic proteins induces the release and activation of proapoptotic proteins Bax and Bid. These interactions cause the leaving of cytochrome c and the apoptosis induction even without transcriptional activation of proapoptotic p53 target genes. The direct apoptosis induction under the p53 influence, perhaps, is the first and extremely rapid response to multiple injuries. The second wave of apoptosis occurs in 6–7 hours and is connected with the transcriptional activity of p53 in the nucleus [3].

In the case of diabetes in response to DNA integrity damage under the action of reactive oxygen species and $ONOO^-$ PARP-1, whose role for this disease was much more complex and diverse, is activated. Thus, it was

shown that glycolysis enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) moves to the nucleus in case of early apoptotic signals, and backwards when such signals disappear. In the nucleus GAPDH can poly-(ADP-ribosyl)ate, losing its enzymatic activity. Inhibition of GAPDH, alongside with high content of glucose in the cell, results in the accumulation of glucose catabolism intermediates in the glycolytic pathway, its splitting to the stage of glyceraldehyde-3-phosphate formation. Consequently, the alternative pathways, such as glucose catabolism polyol and hexosamine are activated, precursors and protein nonenzymatic glycosylation products are accumulated, protein kinase C, which ultimately enhances the pathological changes in diabetes, are activated. The observed ability of PARP-1 to activate the factor NF-κB in the independent from the enzymatic activity way of the enzyme way also enhances the development of inflammatory processes in tissues, even under the absence of DNA damage. The processes based on the pathogenesis of diabetic complications are connected with PARP over activation [5].

The biological effect of the Galega officinalis extract under the condition of diabetes mellitus appears in the reduction of proapoptotic p53 protein and the PARylated proteins content in leukocytes shows the inhibition of genetically programmed death of immune cells in the case of their application. The determined GOE nonalkaloid-containing fraction anti-apoptotic effect may be caused by the presence of flavonoids in the investigated extract, which gives antioxidant effect, since numerous studies show a protective effect of antioxidants in the process of apoptosis [4, 9].

The established reduction of lectin-induced leukocytes aggregation under conditions of diabetes can be caused on one hand by increase in the activity of the endogenous cellular surface sialidase (NEU-3) [1], and on the other hand by the exposure of immature membrane epitopes to the cell surface in response to the loss of surface membrane during cytoplasmic membrane blebbing, that is characteristic of apoptotic cells [12].

The administration of Galega officinalis extract nonalkaloid-containing fraction to diabetic animals causes the increase of the content of the sialic acid residues which are linked α(2→3) and α(2→6) glycosidic bond with the subterminal surface glycoconjugates remains of rats leukocytes. The admitted changes show the normalization of the leukocytes functional state, since it is known that desialidation of surface glycoconjugates under the action of sialidase occuring during the activation of leukocytes [14] shows the decline of apoptosis manifestations under the condition of the researched pathology. The literature contains data, which show the effect of extracts of some medicinal plants on the activity of the trans-sialidase [2].

CONCLUSIONS

The application of GOE nonalkaloid-containing fraction under conditions EDM causes the increase of the sialic acid linked to subterminal galactose (α2→6)- and (α2→3) – bond and reduces the number of leukocytes containing p53 protein and PARylated proteins to the level of control data. The results obtained confirm the corrective influence of the investigated extract on the structural and functional state of leukocytes. The obtained biological effect of the Galega officinalis extract nonalkaloid-containing fraction from one side may be caused by the presence of biologically active compounds in its content that exhibits the antioxidant action and thus inhibits the development of oxidative stress; on the other hand GOE influences the activity of enzymes involved in the cleavage or the transfer of sialic acid residues.

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