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O.Yu. Kushnir, I.M. Yaremii, O.I. Petryshen
Bukovinian State Medical University, Chernivtsi

ANTIOXIDANT ACTION OF MELATONIN IN THE KIDNEY OF ALLOXAN DIABETIC RATS

e-mail: kushnir@bsmu.edu.ua

This study investigated the possible protective effects of melatonin as an antioxidant against alloxan-induced diabetic kidney injury in rats. The introduction of melatonin to alloxan diabetic rats is conducive to a decrease in them of the level of basal glycemia, as well as – a stabilization of the indices of the body's antioxidant defense disturbed namely activities of glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, content of malonic dialdehyde and glutathione in rats kidney. Melatonin not only neutralizes reactive oxygen species, but also acts through the stimulation of several antioxidative enzymatic systems in kidney of alloxan diabetic rats.

Key words: antioxidative system, melatonin, alloxan diabetes, kidney, rats.

О.Ю. Кушнір, І.М. Яремій, О.І. Петришен

АНТИОКСИДАНТНА ДІЯ МЕЛАТОНІНУ У НИРКАХ ЩУРІВ З АЛОКСАНОВИМ ДІАБЕТОМ

У статті розкрито можливий захисний ефект мелатоніну як антиоксидантного засобу проти викликаних алоксаном порушень у нирках щурів. Ін'єкції мелатоніну діабетичним щурам призвели до зниження в останніх рівня базальної глікемії, так само як і до нормалізації показників антиоксидантної системи захисту, а саме активності глутатіонредуктази, глутатіонпероксидази, глюкозо-6-фосфатдегідрогенази, вмісту малонового альдегіду та відновленого глутатіону в нирках щурів. Мелатонін не тільки нейтралізував дію активних форм оксигену, але й сприяв активації ферментативної активності систем антиоксидантного захисту в нирках щурів з алоксановим діабетом.

Ключові слова: антиоксидантна система, мелатонін, алоксановий діабет, нирки, щурі.

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Type 1 diabetes mellitus is characterized by autoimmunity against pancreatic β cells, resulting in their destruction and the patients' subsequent dependency on lifelong insulin replacement. Such patients have many complications, including cardiovascular, renal, and retinal disorders [14]. Diabetes is a disease which disturbs the glycemic control and the antioxidant metabolism disorder plays a role in the development of the clinic state.

Diabetes mellitus can damage the eyes, kidneys, nerves and heart. Microvascular and macrovascular disorders are the leading causes of morbidity and mortality in diabetic patients [13]. Hyperglycemia can increase the indicators of lipid peroxidation and oxidative stress in which free radicals have the main role in the pathogenesis of these complications [12]. Therefore, antioxidants which combat oxidative stress should be able to prevent and repair free radicals induced damages. Although free radicals contribute to kidney damage, atherosclerosis, diabetes, heart disease, nephrotoxicity and hepatotoxicity; however, clinical trials do not uniquely confirm a substantial impact on diabetic damage [8].

Alloxan diabetes was reported to induce oxidative stress and generates reactive oxygen species (ROS) [9]. In the presence of intracellular thiols, especially glutathione, alloxan generates ROS in a cyclic redox reaction with its reduction product, dialuric acid. Autoxidation of dialuric acid generates superoxide radicals, hydrogen peroxide and, in a final iron-catalysed reaction step, hydroxyl radicals. These hydroxyl radicals are ultimately responsible for the death of the beta cells, which have a particularly low antioxidative defence capacity, and the ensuing state of insulin-dependent "alloxan diabetes".

Melatonin (N-acetyl-5-methoxytryptamine) is the major product of the pineal gland, which functions as a regulator of sleep, circadian rhythm, and immune function. Melatonin and its metabolites have potent antioxidant/anti-inflammatory properties, and they have proven to be highly effective in a variety of disorders linked to inflammation and oxidative stress [2]. In general, animals and humans studies documented that short-term use of melatonin is safe, even in extreme doses. Similarly, randomized clinical studies indicate that long-term melatonin treatment causes only mild adverse effects comparable to placebo. [1].

The purpose of the study was to determine the influence of melatonin on basal glucose, malonic dialdehyde, reduced glutathione levels, glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase activities in the kidney of alloxan diabetic rats.

Materials and methods. Study was performed in compliance with the Rules of the work using experimental animals (1977) and the Council of Europe Convention on the Protection of Vertebrate Animals used in experiments and other scientific purposes (Strasbourg, 1986). It was performed according to directions of International Committee of Medical Journals Editors (ICMJE), as well as "Bioethical expertise of preclinical and other scientific research performed on animals" (Kyiv, 2006). Diabetes was induced in male Wistar rats by single i.p. injection of alloxan (170 mg/kg). Four days after diabetes induction, rats were divided into diabetic (untreated) and melatonin-diabetic group (10 mg/kg, daily and orally for one week) [2]. Among diabetic rats were rats with preserved normoglycemia (impaired glucose tolerance – IGT) and rats with diabetes mellitus (DM) basal glucose (BG) level ≥8.0 mmol/l. Blood was taken from the tail vein evaluate the BG level with the use of OneTouchUltra (LifeScan, USA). Rats were sacrificed under light anesthesia at the twelfth day from the beginning of the experiment. The kidney tissue was removed, rinsed in saline, blotted, weighed and homogenized. The homogenate, 5 % in ice-cold 0.25 mM tris-HCl-buffer (pH 7.4), was made using a homogenizer. The supernatant of the homogenate, prepared by ultracentrifugation for 10 min at 3000g/min was used for measurement of activities of enzymes. Oxidant status was assessed by measuring of malonic dialdehyde (MDA), reduced glutathione (GSH) levels, glutathione reductase (GR), glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase (G-6-PhD) activities.

In the process of oxidative modification of proteins in the radicals of the aliphatic amino acid residues, aldehyde and ketone groups are formed. They interact with 2.4-dinitrophenylhydrazine to form 2.4-dinitrophenylhydrazones with a specific absorption spectrum. Aldehyde- and keto-derivatives which are neutral in nature are determined at a wavelength of 370 nm, alkali – at 430 nm [5].

The method of MDA determination [4] is based on a spectrophotometric determination of the trimetinic colored complex formed from the MDA interaction with thiobarbituric acid.

The spectrophotometric/microplate reader assay method for GSH involves oxidation of GSH by the sulfhydryl reagent 5.5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm [15].

The activity of GR was determined [15] by the rate of glutathione recovery in the presence of $NADPH_2$. The GR activity was determined in a surface solution of centrifugate (1500 g, 10 min) by decreasing the amount of $NADPH_2$.

The activity of GP was determined [15] by the amount of oxidized glutathione formed from reduced glutathione in the detoxification of hydrogen peroxide in the glutathione peroxidase reaction (modification by Gerus I.V., Grigorieva N.P., Meschyshyn I.F.).

The investigation of G-6-PhD activity was made [14] spectrophotometrically according to increase of the optical density at 340 nm, which is due to the rise in the number of $NADPH_2$ in the process of enzymatic reaction.

Total protein determination (according to Lowry). It is performed according to the process described by V. Gudumac and coauthors [4].

Statistical analysis was performed using Statistica 10 (StatSoft Inc). Prior to analysis, Shapiro-Wilk test was used to assess the normality of the group data. According to the criterion, the samples distributions differed from normal distribution. Given these, use of the Mann-Whitney test was considered sufficient for valid conclusions to be made. Differences were considered to be statistically significant if $p<0.05$.

Results of the study and their discussion. The BG level was increased up to 120 % from baseline on the 4th day of the experiments in the animals which were injected by alloxan monohydrate. In the diabetic rats (untreated) this index continued to rise over a one week period starting on the 4th day of the experiments and was on 150 % higher compared with baseline. Melatonin insertion reduced (but not normalized) the level of BG 1.8 times compared with the index DM animals.

The MDA (tab.1) levels were found to be higher on 60 % in DM group and on 23 % in IGT group respectively than in control. So, the lipid peroxidation was increased in diabetic kidney. Melatonin partly prevented diabetes-induced increase in MDA levels in kidney.

Table 1

Changes of the antioxidant defence in kidney of diabetic rats, (n=6, x±S$\bar{x}$)

Indices / Groups	MDA, mkmol /g	G-SH, mkmol/g	GPx, nmol/min×mg	G-6-PhD, nmol/min×mg	GR, nmol/min×mg
1.Control group	25.0±1.23	4.2±0.03	145.8±9.42	4.2±0.10	5.2±0.12
2. Diabetes mellitus group	40.2 ± 0.68^{a}	2.2 ± 0.04^{a}	120.2 ± 8.21^{a}	2.3 ± 0.09^{a}	3.0 ± 0.14^{a}
3. Diabetes mellitus + melatonin group	23.5 ± 0.47^{b}	4.3 ± 0.03^{b}	150.2 ± 8.34^{b}	5.6 ± 0.12^{b}	5.7 ± 0.16^{b}
4. Impaired glucose tolerance group	$30.6\pm0.43^{a,b}$	$6.4\pm0.04^{a,b}$	$168.0\pm10.1^{a,b}$	$8.0\pm0.14^{a,b}$	$7.2\pm0.18^{a,b}$
5. Impaired glucose tolerance + melatonin group	$23.7\pm0.45^{b,c}$	$4.4\pm0.02^{b,c}$	$155.0\pm8.8^{b,c}$	$4.3\pm0.08^{b,c}$	$5.3\pm0.15^{b,c}$

Note: 1. a, b, c – changes are reliable ($p<0.05$). 2. a – concerning control group of rats; b – concerning group of rats with diabetes mellitus; c – concerning group of rats with impaired glucose tolerance.

It let to decrease this index in DM and IGT groups of diabetic animals on 41 % and 22 % respectively compared with control what meant the normalization of MDA level.

Diabetes mellitus produces disturbances in the lipid profile of body making the cells more susceptible to lipid peroxidation. Experimental studies show that polyunsaturated fatty acids in cell membrane are extremely prone to attack by free radicals due to the presence of multiple bonds. Lipid hyperperoxides through intermediate radical reactions produce such fatty acids that generate highly reactive and toxic lipid radicals that form new lipid hyperperoxides. A critical biomarker of oxidative stress is lipid peroxidation which is the most explored area of research when it comes to ROS. MDA are formed as a result of lipid peroxidation that can be used to measure lipid peroxides after reacting it with thiobarbituric acid.

Diabetes induces alterations in activity of enzymes GPx and GR (tab.1). These enzymes are found in cell that metabolizes peroxide to water and converting glutathione disulfide back into glutathione. Any alteration in their levels will make the cells prone to oxidative stress and hence cell injury.

On the other hand GR, GPx, G-6-PhD activities also depend on the presents of hyperglycemia. In DM group of rats activities of GR, GPx, G-6-PhD were decreased on 42 %, 18 %, 46 % respectively compare with control rats. We have found the level of GSH lover by 48 % in DM group of animals compared with control. These results are consistent with the degenerative role of hyperglycemia on cellular reducing equivalent homeostasis and antioxidant defense, and provide further evidence that pharmacological intervention of antioxidants may have significant implications in the prevention of the prooxidant feature of diabetes and protects redox status of the cells. ROS reacts with some amino acid, producing anything from modified, denatured and non-functioning proteins that in further may be responsible for oxidative stress. Diabetic hyperglycemia, by the process of free radical production, causes protein glycation and oxidative degeneration. The degree of such protein glycation is estimated by using some biomarkers such as glycated hemoglobin. Reduction of enzyme activities is possible due to glycosylation.

In the group of rats with preserved normoglycemia (IGT) activities of GR, GPx, G-6-PhD were increased on 38 %, 15 %, 90 % respectively compare with control rats. Increase of G6PhD activity in condition of diabetes with IGT is probably a compensatory reaction aimed to reduce of ROS. It was found that the level of GSH increased by 54 % compared with control. $NADPH_2$ reducing equivalents (that are produced in this reaction) are used for regeneration of glutathione from its oxidized form due to action of $NADPH_2$-dependent glutathione reductase. Glutathione neutralizes ROS, both directly and through GPx. Melatonin injections were helpful for normalization this indexes under study. It let to decrease the activities of GR, G-6-PhD, as well as content of GSH on 24 %, 45 %, 31 % respectively compared with IGT group of rats. Same time we observed that in IGT group the content of MDA was increased (as we mentioned before). That meant the intensification of lipid peroxidation processes even in diabetic group with preserved glycaemia while melatonin injections helped to decrease this index to normal level. Therefore, introduction of this antioxidant decreased MDA content (on 22 %) that the last one did not differ from the control. In this case, melatonin probably increases use of glucose for regeneration of $NADPH_2$ and aerobic oxidation of glucose that indicate an acceleration of antioxidative protection and energy production in kidney of diabetic rats.

Melatonin besides being safe, lowered the blood glucose significantly without any hypoglycemic effect on their normoglycemic counterparts.

Possible melatonin inhibits glycation by reducing the generation of reactive carbonyl or dicarbonyl groups either from fructosamine or glucose, probably due to stimulation of glucose transport to skeletal muscle cells and preventing of ROS formation in conditions of hyperglycemia.

It was detected, that melatonin stimulates glucose transport to skeletal muscle cells via insulin receptor substrate-1/phosphoinositide 3-kinase (IRS-1/PI-3-kinase) pathway, which implies, at the molecular level, its role in glucose homeostasis and possibly in diabetes [8]. Endogenous melatonin level may contribute to the incidence and/or development of diabetes. In addition, melatonin may increase a plasma concentration of leptin in mice [3] and there are findings that terminally ill insulin-deficient rodents with uncontrolled diabetes due to autoimmune or chemical destruction of beta-cells were made hyperleptinemic by an adenoviral transfer of the leptin gene. Within approximately 10 days, their severe hyperglycemia and ketosis were corrected. Despite the lack of insulin, moribund animals resumed linear growth and appeared normal. Inhibition of gluconeogenesis by suppression of hyperglucagonemia and reduction of hepatic cAMP response element-binding protein, phoshoenolpyruvate carboxykinase and peroxisome proliferator-activated receptor-gamma-coactivator-1alpha may explain the anticatabolic effect.

Moreover, earlier [8] we investigated Langergans islends in diabetic rats and recorded histomorphological alterations: their pancreatic share reliably decreased by 55 %, number and percentage of beta-cells with necrosis decreased by 90 % and 97 % respectively compared with the control. Melatonin treatment caused a sharp decrease in the elevated serum glucose and partial regeneration/proliferation of beta-cells. It was concluded that the hypoglycemic action of melatonin could be partly due to amelioration in beta-cells of pancreatic islets.

Alteration in function and structure of antioxidant protein enzymes may also be due to nonenzymatic glycation such that detoxification of free radicals is effected enhancing oxidative stress in diabetes [6].

The actions of melatonin on radical metabolizing/producing enzymes may be mediated by the Keap1-Nrf2-ARE pathway. Beyond its direct free radical scavenging and indirect antioxidant effects, melatonin has a variety of physiological and metabolic advantages that may enhance its ability to limit oxidative stress [11].

Conclusion

We have found reduction of antioxidative defense in the kidneys of diabetic rats by observing of decrease activities of glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase and level of reduced glutathione, while malonic dialdehyde content was increased.

Melatonin not only neutralizes reactive oxygen species, but also acts through the stimulation of several antioxidative enzymatic systems in kidney of alloxan diabetic rats by normalization of enzymes activities and levels of malonic dialdehyde and reduced glutathione.

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