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MELATONIN IMPROVES SPLEEN HISTOPHYSIOLOGY OF RATS WITH DIET-INDUCED OBESITY: CHRONOTHERAPY APPROACH

One of the most commoncharacteristics of obesity is the development of a systemic low-grade proinflammatory state in the entire body, including the immune organs. Spleen enlargement during diet-induced obesity contributes to the development of chronic inflammation. Melatonin due to immunomodulatory, antioxidant, and systemic metabolic rolesis proposed to be an effective candidate for anti-obesity therapy. As immune systems demonstrate pronounced circadian rhythmicity and immune cells have different types of melatonin receptors, a chronotherapeutic approach might be used to choose the most effective regimes of melatonin administration for the correction of obesity-provoked damage to the spleen. Thus, the main goal of our research was the analysis of the rats' spleen histophysiology during the development of high-calorie diet-inducedobesity (HCD) after administering melatonin daily at different times (morning or evening). Melatonin was administered by gavage for 7 weeks in the dose of 30 mg/kg 1 h before lights-off (HCD ZT11, M ZT11, evening), or 1 h after lights-on (HCD ZT01, M ZT01, morning). For assessment of the morpho-functional state of the spleen,the histopathological evaluation of red and white pulp in different zones of lymphoid follicles was implemented. It was observed that obesity development wasaccompaniedbyhyperemia and vessel dilatation in the red pulp; while in the white pulp notable deformation of germinal centers and destroyed borders between zones of lymphoid follicles were noticed.The HCD group demonstrated a decrease inthe relative amount of the white pulp, the cross-sectional area of germinal centers, and the cross-sectional area of the marginal zone; whilethe increased relative amount of red pulp and marginal zone/germinal centers ratiowere detected compared with control. Melatonin administration to obese rats increases the relative amount of the white pulp (HCD ZT11 group), the cross-sectional area of germinal centers (HCD ZT01 and HCD ZT11 groups), and the cross-sectional area of the marginal zone (HCD ZT11 group), and decreasesmarginal zone/germinal centers ratio (HCD ZT01 group) in comparison with the HCD group.Also,it was demonstrated that a choice between the morning or evening regimes of the melatonin treatment did not affect the histophysiology of the spleen in rats receivingthe standard diet (M ZT01 and M ZT11 groups). These results indicate that melatonin can be considered to be a powerful potential therapeutic agent for the amelioration of obesity-induced changes in the spleen.

Keywords: chronobiology, high-calorie diet-induced obesity, white pulp, red pulp, germinal centers, marginal zone.

Introduction

The morphological state of the spleen depends on metabolic disorders in the body [16]. Previous studies have reported an association between obesity and an enlarged spleen or non-alcoholic fatty liver disease [3, 27]. The spleen, as a peripheral organ of the immune system, reacts to any immunopathological process in the body, ensures erythrocyte homeostasis, participates in the effector phase of the humoral immune response and hematopoiesis, metabolism, and it is one of the main blood depots [14]. The spleen affects glucose-induced insulin secretion from pancreatic islets and simultaneously modulates glucose tolerance [20]. Clinical data show that the incidence of diabetes is significantly higher in patients who have undergone partial pancreatectomy and splenectomy than in those who have undergone pancreatectomy alone. In addition, the spleen affects glucose homeostasis in obesity [11]. The mechanism of splenomegaly in obesity remains unclear [26, 5, 12]. One explanation for this is that the spleen, as an organ of the immune system, can become enlarged as a result of the chronic inflammation associated with obesity [2, 15]. This inflammatory state is the result of overproduction of inflammatory mediators such as tumor necrosis factor-α and interleukin-6 and a decrease in the production of anti-inflammatory interleukin-10 [1, 13, 8, 9]. With an unbalanced diet and failure to maintain a healthy lifestyle, a significant decrease in lymphocytes, coarsening of the reticular fibers of the spleen stroma, and mucoid swelling of the vessel walls of the microcirculatory bed are observed [25]. An excess of dietary unsaturated fatty acids in the range of 800-1200 mg/kg causes significantoxidative stress, which provides a possible pathway for splenocyte apoptosis [22]. The degree of the detected disruptionsdepends on the strength of the high-calorie load and is a manifestation of the body's general adaptive response. In addition, an increase in the mass of the spleen, but a decrease in the cellularity of the organ, was found in animals that consumed a high-calorie diet [17]. Splenomegaly without an increase in the cellularity in obesity may be a consequence of a prolonged efferent phase during the activation of antibody production [28, 24]. Such indicators may indicate possible exhaustion of compensatory and adaptive mechanisms and homeostatic imbalance.

Melatonin is a multifunctional signal molecule with a pronounced immunomodulatory function [6]. The best-characterisedfunction of melatonin is the regulation of the circadian rhythm of the whole body. Desynchronosisof an endogenous circadian rhythm with external conditions leads to depression, decreased immunity, and, as a result, neurological, autoimmune, metabolic, endocrine, and oncological diseases. The basis of the development of all these diseases is the failure of endocrine, neuronal, and immune regulatory mechanisms. Currently, melatonin is receiving special attention in the search for effective and safe pharmacological correctors for pathological conditions, in particular, obesity [4]. Its biological significance is determined by its regulatory influence on all types of biological rhythms that underlie life processes and occur at all levels of organization: cellular, tissue, organ, and system. Melatonin regulates the rhythmicity of lymphoid organs through the activation of nuclear and membrane melatonin receptors [19].

Therefore, the main aim of our research was to analyze the histophysiology of rat's spleen (relative amount of white and red pulp, the cross-sectional area of germinal centers and marginal zone in the white pulp, marginal zone/germinal centers ratio) after different melatonin regiments (morning and evening) during the development of high-calorie-diet-induced obesity.

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Materials and methods

White nonlinear male rats (110±10 g bodyweight) were used in this study. The light cycle was set as 12-h light and 12-h darkness, with lightsoff at 19:00 (ZT12). All experiments on animals were carried out in compliance with the international principles of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (European Convention, Strasburg, 1986), Article 26 of the Law of Ukraine "On the Protection of Animals from Cruelty" (No.3447-IV, February 21, 2006) as well as all norms of bioethics and biological safety.

During the first week, all animals received standard rodent chow (15,3 kJ•g^{-1}). Food and water were available *ad libitum*. Animals were kept under standard housing conditions with constant temperature and humidity. On the 8th day, rats were divided into two groups: control animals received standard chow for 13 weeks and experimental rats received a high-calorie diet (HCD, 22,4 kJ•g^{-1}), consisting of standard chow (60 %), lard (10 %), eggs (10 %), sugar (9 %), peanut (5 %), dry milk (5 %) and vegetable oil (1 %) [10]. To confirm the development of obesity, animals were weighed once a week until the average body weight gain reached a significant difference of at least 30 %. Then animals were classified as having normal body mass (Control) and those with developed obesity (HCD). Rats of control and HCD groups were further divided into three subgroups each:

1. Control group – no administration of melatonin, standard diet (15,3 kJ•g^{-1});
2. Group M ZT01 – melatonin in the morning (1 hour after light-on), standard diet (15,3 kJ•g^{-1});
3. Group M ZT11 – melatonin in the evening (1 hour before light-off), standard diet (15,3 kJ•g^{-1});
4. Group HCD – no administration of melatonin, high-calorie diet (22,4 kJ•g^{-1});
5. Group HCD ZT01 – melatonin in the morning (1 hour after light-on), high-calorie diet (22,4 kJ•g^{-1});
6. Group HCD ZT11 – melatonin in the evening (1 hour before light-off), high-calorie diet (22,4 kJ•g^{-1}).

Melatonin (Alcon Biosciences, USA) was diluted in drinking water and administered daily by single oral 2 mL gavage in the dose of 30 mg/kg bodyweight. The administration lasted for 7 weeks. Melatonin treatment began duringthe 6th week after the inductionof obesity.

For the treatment of many experimental disease models [23] and also in the case of clinical trials [7], the use of different doses, methods, and times of melatonin administration had been employed. In our experiments, the lowest dose of melatonin was chosen, at which it was observed both a simultaneous decrease in weight gain of obese rats and the appearance of beige adipocytes, as we are interested in obesity therapy through beige and brown adipocyte activation.

On the last day of the experiment, the animals were sacrificed by carbon dioxide asphyxiation and decapitated, and then spleen tissue samples were isolated and weighted.

A histopathological examination was performed to characterize the morphology and functional status of the spleen. Fragments of the spleen in the size of 5 × 5 mm were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 72 h, dehydrated, embedded into the paraffin, and cut into 7 μm sections according to standard procedures. Slides were stained with Bemer'shematoxylin and eosin (H&E).

Quantitative determination was performed using digital microphotographs. All captures were obtained using a light microscope BX41 (Olympus, Japan) with a4×objective lens. Microphotographs were taken using the DP20 (Olympus, Japan) digital camera andanalyzed with the QuickPHOTO MICRO software (Promicra, Czech Republic).

Histological evaluation was performed by accounting relative amounti of the white and red pulp, the cross-sectional area of germinal centers, and the marginal zone in the white pulp, marginal zone/germinal centers ratio. All parameters were measured with the ImageJ software (National Institutes of Health, USA). For morphometric analyses we analyzed 2 slides from each experimental animal. The number of observations for each group was 50 per each morphometric parameter.

Statistical data analysis was performed using Statistica 6.0 (StatSoft, USA) and Microsoft Excel 2010 software (Microsoft, USA). The obtained data were presented as means ± standard error of the mean (SEM). The distribution of data was assessed with the Shapiro-Wilk normality W-test. Since the analyzed distributions were considered normal, we used one-way ANOVA followed by Dunnet`s multiple comparisontest to evaluate the differences between the means. The differences with the probability of the null hypothesis of $p < 0.05$ were considered significant.

Results and Discussion

Normally (**Fig. 1, control group**), the spleen of rats is surrounded by a capsule of fibrous, dense connective tissue. It has numerous elastic and collagen fibers, and smooth muscle cells, and it is covered with mesothelium on the outside. Trabeculae depart from the capsule into the inner part of the organ, and blood vessels are located in their thickness. In the spleen of rats, two functional zones can be clearly distinguished –the white and red pulp. The white pulp is represented by spherical formations that are made of lymphocytes surrounding arterioles. Clusters of T-lymphocytes form a periarteriolar lymphocyte sheath. B-lymphocytes form lymphoid follicles that have clearly defined germinal centers and mantle zone surrounded by a loosely distributed marginal zone. The red pulp is made up of sinusoidal capillaries and splenic cords located between them, which anastomose with each other. Splenic cords are clusters of blood cells: erythrocytes, macrophage cells, and leukocytes, including T- and B-lymphocytes at various stages of differentiation. The stroma of the red pulp is made of reticular tissue.

In the HCD group (**Fig. 1**), the share of thewhite pulp volume decreases, and the number of cells in it also decreases. The mass of the spleen increases as a result of swelling of the parenchyma and the overfilling of vessels with the blood. The number of secondary lymphoid nodules in the part of the white pulp increases, and the germinal centers aredeformed; the boundaries between the zones become unclear. In the organ parenchyma, the number of macrophages, monocytes, plasma cells, and myeloid cells increases. Macrophages are in an active state, the cytoplasm is filled with remnants of cells, hemosiderin, and erythrocytes. In the red pulp of obese rats, vascular disorders manifested in the form of swelling of the splenic stroma and blood vessels, dilation of the marginal sinuses, and hyperemic blood vessels are also observed. In the white pulp, the zone of lymphoid follicles becomes looser and lighter than normal, and the number of lymphoid elements decreases compared to the control group.

Melatonin administration to rats on a standard diet (group M ZT01 and M ZT11) did not induce morphological changes in any applied regime.

Melatonin administration to rats with a high-calorie diet (group HCD ZT01 and HCD ZT11) improved the morphological state of both white and red pulp of the spleen: the

amount of blood in the venous sinuses of the spleen decreased. Macrophages that are completely filled with hemosiderin and elements of other cells are less common. The nuclear envelope of lymphocytes hadclear contours, but the cytoplasm was clear and did not contain organelles with any signs of swelling and damage. The lumen of the capillaries wasnarrowed, and the wall of the venous sinuses was not thickened.

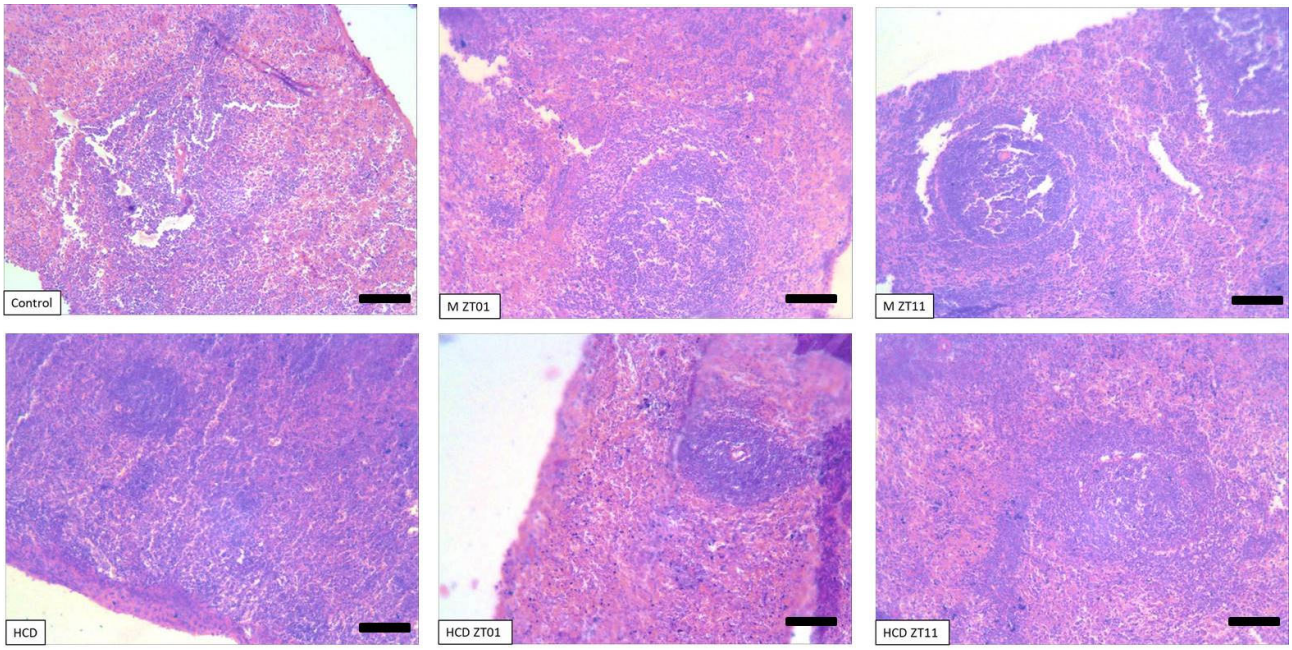

Fig. 1. Microphotographs of rats' spleen sections: H&E staining; scale bar: 50 μm

Morphometric analysis of the obtained data demonstrated a significant decrease by 34% inthe relative amount of the white pulp in obese rats after consuming a high-calorie diet compared to the control (**Fig. 2**). Melatonin administration during HCD-induced obesity prevented the decrease of the relative amount of the white pulp after evening interventions: this parameter increased by 22% in comparison to the HCD group, but it was still low in comparison with control – in HCD ZT11it decreased by 20% and in the HCD ZT01- by 26%. The relative amount of the white pulp in the M ZT01 and M ZT11 groups did not differ from the control value. There was no observed difference in the relative amount of white pulpbetween morning and evening regimes of melatonin administration in the HCD ZT11 and HCD ZT01 groups.

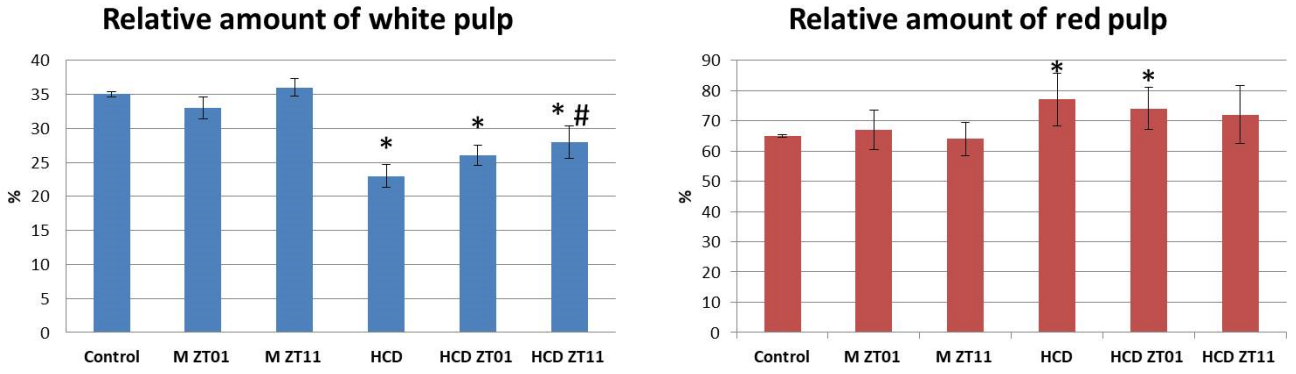

Fig. 2. Relative amount of the white and red pulp of the spleen

Notes: * – difference between the control and experimental groups are significant at $p \leq 0.05$;
– difference between the HCD group and HCD ZT01, HCD ZT11 is significant at $p \leq 0.05$;
The number of observations for each group was 50

HCD-induced development of obesity resulted in a significant increaseof the relative amount of the red pulp by 19%, compared to the control group (**Fig. 2**). High level of the relative amount of red pulp also was fixed in HCD ZT01: itincreased by 14% in comparison with the control group. The relative amount of the red pulp in the HCD ZT11 group hasan intermediate value: its level did not significantly differ from the control and the HCD groups. Also, morning and evening melatonin regimes did not influence the relative amount of the red pulp in rats consuming a standard diet. There was no observed difference in the relative amount of

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red pulpbetween morning and evening regimes of melatonin administration in the HCD ZT11 and HCD ZT01 groups.

Also, morphometric parameters of lymphoid follicles have been analyzed (**Fig. 3**). The cross-sectional area of germinal centers in obese rats (HCD group) decreased by 64% in comparison with the control. Administration of melatonin in both morning and evening regimes to rats with HCD (groups HCD ZT01 and HCD ZT11) resulted in a 2-fold increasein the cross-sectional area of germinal centers in comparison with the HCD group. Their values reached the control level. Interestingly, the morning administration of melatonin to rats with a standard diet increasedthe cross-sectional area of germinal centers by 74% in comparison with the control, while evening administration of melatonin to rats without obesity did not influence this parameter.

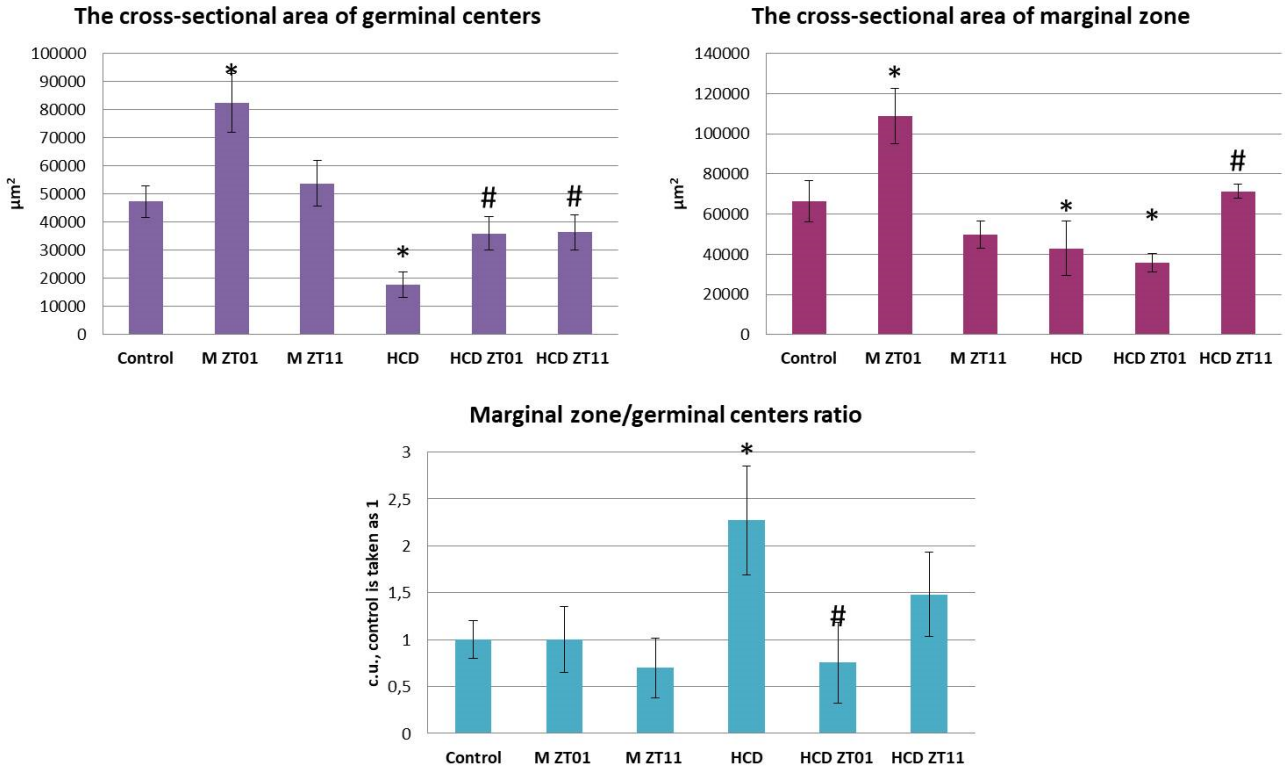

Fig. 3. Morphometric parameters of lymphoid follicles: the cross-sectional area of germinal centers, the cross-sectional area of marginal zone, and marginal zone/germinal centers ratio

Notes: * – difference between the control and experimental groups is significant at $p \leq 0.05$;
– difference between the HCD group and HCD ZT01, HCD ZT11 is significant at $p \leq 0.05$;
The number of observations for each group was 50

The cross-sectional area of the marginal zone (**Fig. 3**) in the HCD group increased by 46% comparedto the control. In case of the cross-sectional area of the marginal zone different regimes of melatonin administration affected obese rats differently: the morning regime (HCD ZT01 group) did not causeany rise in this parameter, but it is still decreased by 47% in comparison with the control. However, the evening regime of melatonin administration induced an increase of the cross-sectional area of the marginal zone by 66% compared with HCD and did not significantly differ from the control. Like in the case with the cross-sectional area of germinal centers, the morning regime of melatonin administration affected the cross-sectional area of the marginal zone in rats with a standard diet (group M ZT01) increasing it by 63% comparedto the control. Evening melatonin administration regimes did not affectthecross-sectional area of the marginal zone in rats consuming a standard diet.

The marginal zone/germinal centers ratio parameter (**Fig. 3**) demonstrates interrelationbetween the different zones of lymphoid follicles and changes in a mixed population with T-, B- lymphocytes and macrophages. The marginal zone/germinal centers ratio in the HCD group increased 2.3-fold compared to the control. The morning regime of melatonin administration in obese rats decreased the marginal zone/germinal centers ratio by 67% compared with the HCD group and reached control level. Whilethe marginal zone/germinal centers ratioin the HCD ZT11 group has an intermediate value: its level did not significantly differ from both control and the HCD group.Also, morning and evening melatonin regimes did not affectthemarginal zone/germinal centers ratio in rats consuming a standard diet (groups M ZT01 and M ZT11).There was no observed significant difference in the marginal zone/germinal centers ratiobetween morning and evening regimes of melatonin administration in the HCD ZT11 and HCD ZT01 groups.

Taking into account the results of morphological observations and morphometric analysis, melatonin was shown to have the corrective effect on the spleen histophysiologyduring obesity development without any negative influence on the spleen of rats consuming a standard diet.

The potential mechanisms of melatonin action on amelioration spleen function during obesity development are

reduction of oxidative stress and activation of splenocyte proliferation [21]. Lymphocyte proliferation can be transduced throughmembrane melatonin receptors Mel1b, Mel1c, andnucleus receptors RORα/RORγ for T-lymphocyte; and throughmembrane melatonin receptors Mel1a, Mel1c, and RORα B-lymphocyte proliferation [29]. In Alloxan-model of diabetes it was shown that melatonin (melatonin treatment intraperitoneally at a dose of 10mg/kg^{-1} per day for 15 days) inhibited the production of proinflammatory cytokines (serum IL-1β) and tissue mast cell accumulation in the spleen, thymus, and lymph node [18]. Also melatonin induces M2 polarization of macrophages through STAT3 pathway [30].

Conclusions

Daily administration of exogenous melatonin (30 mg/kg for 7 weeks) in different regimes improves the morpho-functional state of the spleen during high-calorie diet-induced obesity that manifested in decreased swelling of the parenchyma and hyperemic blood vessels in the red pulp, reorganization of the different zones of white pulp. This effect developswithout any pathological influence on the spleen of rats that consumed a standard diet. It is shown that the morning administration of melatonin induced the cross-sectional area of the germinal center and the marginal zone/germinal centers ratio wererestored back to the control values; whileevening administration inducedchanges of the cross-sectional area of the marginal zone, the cross-sectional area of the germinal center, and the relative amount of the white pulp as compared with obese rats.

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МЕЛАТОНІН ПОКРАЩУЄ ГІСТОФІЗІОЛОГІЮ СЕЛЕЗІНКИ ЩУРІВ З ІНДУКОВАНИМ ОЖИРІННЯМ: ХРОНОТЕРАПЕВТИЧНИЙ ПІДХІД

Однією з ознак ожиріння є розвиток системного прозапального стану низького рівня в усьому організмі, включаючи органи імунної системи. Збільшення селезінки при ожирінні, спричиненому споживанням висококалорійної дієти, сприяє розвитку хронічного запалення. Мелатонін завдяки імуномодулюючій, антиоксидантній та метаболічній функціям вважається ефективним кандидатом для терапії ожиріння. Оскільки імунна система демонструє виражену циркадну ритмічність, а імунні клітини мають різні типи рецепторів мелатоніну, то хронотерапевтичний підхід може бути використаний при виборі найбільш ефективних режимів введення мелатоніну для корекції пошкодження селезінки, спровокованого ожирінням. Отже, основною метою нашого дослідження був аналіз гістофізіології селезінки щурів під час розвитку ожиріння, спричиненого споживанням висококалорійної дієти (HCD) після різного часу (вранці або ввечері) щоденного введення мелатоніну. Мелатонін вводили через зонд протягом 7 тижнів у дозі 30 мг/кг за 1 годину до вимкнення світла (HCD ZT11, M ZT11, вечір) або через 1 годину після включення світла (HCD ZT01, M ZT01, ранок). Для оцінювання морфофункціонального стану селезінки використовували патогістологічне дослідження червоної, білої пульпи та різних зон лімфоїдних фолікулів. Розвиток ожиріння супроводжувався гіперемією і розширенням судин червоної пульпи; при цьому в білій пульпі зазначали помітну деформацію зародкових центрів і зруйновані межі між зонами лімфоїдних фолікулів. Група HCD демонструє зменшення відносної кількості білої пульпи, площі поперечного перерізу зародкових центрів і площі поперечного перерізу крайової зони при підвищенні відносної кількості червоної пульпи та співвідношення крайової зони / зародкових центрів порівняно з контролем. Введення мелатоніну щурам з ожирінням збільшує відносну кількість білої пульпи (група HCD ZT11), площу поперечного перерізу зародкових центрів (групи HCD ZT01 і HCD ZT11), площу поперечного перерізу крайової зони (група HCD ZT11) і зменшує співвідношення маргінальної зони / зародкових центрів (група HCD ZT01) порівняно із групою HCD. Також лікування мелатоніном вранці або ввечері не вплинуло на гістофізіологію селезінки щурів, які споживали стандартну дієту (групи M ZT01 та M ZT11). Ці результати показали, що мелатонін можна розглядати як потужний потенційний терапевтичний засіб для відновлення змін селезінки, спричинених ожирінням.

Ключові слова: хронобіологія, індуковане висококалорійною дієтою ожиріння, біла пульпа, червона пульпа, гермінативні центри, крайова зона.