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INFLAMMATION FEATURES OF BROWN ADIPOSE TISSUE OF RATS WITH DIET-INDUCED OBESITY DEVELOPMENT AFTER DIFFERENT REGIMES OF MELATONIN ADMINISTRATION

One of the prominent obesity-related changes is the development of systemic low-grade proinflammatory state. Brown adipose tissue (BAT) may serve as a potential target for activation by melatonin to facilitate heat production and simultaneously stimulate lipolysis during obesity development. At the same time, melatonin is known to have immunomodulatory properties, which are performed via endocrine and paracrine signal pathways in variety cell types (including brown adipocytes) and change significantly during the day. Therefore, it can be used in a wide range of doses and at different times of the day (chronotherapeutic approach). Thus, the main goal of our research was to analyze the inflammation state of brown adipose tissue of rats during high-calorie diet induced-obesity development after different daily melatonin application in different regimes. Melatonin was administered by gavage for 7 weeks in dose 30 mg/kg 1 h before lights-off (HCD ZT11, M ZT11, evening), or 1 h after lights-on (HCD ZT01, M ZT01, morning). Tissue collagen content and leukocyte infiltration levels in BAT, detected by Van Gieson trichrome staining, were used as markers for assessment of BAT inflammation state. Propagation of obesity resulted in the increase of BAT fibrosis level (the relative area occupied by collagen fibers) and tissue leukocyte infiltration in comparison to control rats. BAT fibrosis level after melatonin administrations to obese rats of HCD ZT01 and HCD ZT11 groups decreased to control values. Similar effects were observed in BAT tissue leukocyte infiltration after both regimes (HCD ZT01 and HCD ZT11 groups) of melatonin intake: this parameter decreased significantly, comparing to obese rats, but was still elevated, comparing to controls. At the same time, melatonin treatment in morning or evening regimes did not have any impact on BAT fibrosis propagation and leukocyte infiltration in animals that consumed standard diet (M ZT01 and M ZT11 groups). To sum up, we suggest corrective properties of melatonin in context of chronic low-grade inflammation in obese rats BAT and suppose its wide potential for the therapeutic use combined with virtually absent side effects on BAT histophysiology of non-obese rats.

Keywords: chronobiology, histochemistry, brown adipocytes, fibrosis, leukocyte infiltration, high-calorie diet-induced obesity.

Introduction. Obesity is associated with an increase in size (hypertrophy) and *de novo* formation of white adipocytes (hyperplasia) [1]. Obesity-related changes occur not only in white adipose tissue, but also in brown adipose tissue (BAT). These changes are manifested in several processes, which are collectively named "whitening" of BAT [2]. During "whitening" brown adipocytes dramatically change their morphology to a white adipocytes-resembling phenotype [3]. The increase in adipocytes' volume and tissue mass causes inflammation, further deregulation of adipose tissue and, ultimately, adipose tissue fibrosis [4], which is manifested in obesity and diabetes. The main inflammatory cytokines, whose mRNAs are detected in BAT of obese animals, are TNF α [5], F4/80 antigen – a major murine macrophage marker [6], monocyte chemoattractant protein 1 [7]. High levels of inflammatory molecules can lead to inhibition of insulin-induced glucose uptake and to the development of insulin resistance in BAT [8]. Alternatively, pro-inflammatory cytokines decrease UCP 1 (uncoupling protein 1) expression in brown adipocytes, which results in decline of heat production capacity through different signal transduction pathways, possibly including SIRT1 (Sirtuin 1 – NAD-dependent protein deacetylase) and its negative regulator DBC1 (deleted in breast cancer 1) [9]. In addition, TNF α may serve as a potential candidate for thermogenesis inhibition in brown adipocytes [10]. Another, indirect, mechanism was proposed – pro-inflammatory cytokines recruit inflammation-driven macrophages to BAT, which affects norepinephrine sensitivity of brown adipocytes (the main activation mechanism of UCP 1 production) by inflammasome-mediated pathway. These changes may involve regulation of monoamine oxidase A (a catecholamine degrading enzyme) and norepinephrine transporter, that influence on norepinephrine bioavailability

[11, 12]. The long-term consumption of high-fat diet also results in attraction of lymphocytes and granulocytes to BAT (besides macrophages and monocytes), which promotes pro-inflammatory state in tissue [13, 14].

Like other fibrotic diseases, adipose tissue fibrosis is the accumulation and increased production of extracellular matrix proteins [15]. Several factors, like leukocytosis, enlargement of adipocytes, and overproduction of HIF (Hypoxia-inducible factor) promote deposition of collagen fibers via upregulation of pro-fibrotic genes such as lysyl oxidase, collagens I and III [16, 17]. Fibrosis also decreases flexibility of extracellular matrix and remodeling abilities of adipose tissue, which are associated with comorbidity of metabolic diseases [18].

Melatonin is a multifunctional signal molecule with pronounced immunomodulatory function [19]. In particular, melatonin possesses anti-inflammatory properties in obesity treatment [20]. Melatonin receptors are found in brown adipocytes [21] and in many leukocytes [22]. Main effects of melatonin on BAT are caused by modulation of UCP 1 production and mitochondrial functionality [23]. However, effects of melatonin usage on brown adipose tissue remodeling during obesity and corresponding fibrosis development remain largely unknown.

Therefore, the main aim of our research was to analyze the inflammation state of brown adipose tissue of rats (fibrosis level and tissue leukocytes infiltration) after different melatonin regimens against the background of high-calorie-diet-induced obesity.

Materials and methods. White nonlinear male rats (110 \pm 10 g bodyweight) were used in this study. The light cycle was set as 12-h light and 12-h darkness, with lights-off 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, Strasbourg, 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 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 %) [24]. To confirm the development of obesity, animals were weighted once a week until the average body weight gain reached a significant difference of at least 30 %. Then animals were classified as having the normal body mass (Control) and those with developed of 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 administrations lasted for 7 weeks. Melatonin treatment began at the 6th week after the propagation of obesity.

Different doses, methods and times of melatonin administration were previously shown to be effective in many experimental diseases models [25] and also clinical trials [26]. We chose the lowest dose of melatonin which causes both a decrease in obese rats' weight gain and the appearance of beige adipocytes, as we were interested in obesity therapy through beige and brown adipocytes' activation.

On the last day of the experiment, animals were sacrificed by carbon dioxide asphyxiation and decapitated, and then brown adipose tissue samples were isolated from the interscapular region.

Histopathological examination was performed to characterize the morphology and inflammation status of BAT. Fragments of BAT in the size of 5 × 5 mm were fixed in 4 % paraformaldehyde in 0,1 M phosphate buffer for 72 h, dehydrated, embedded into paraffin and cut into 7 μm sections according to standard procedures.

Tissue fibrosis (estimated as relative area occupied by collagen fibers) and tissue leukocyte infiltration were used as criteria for the assessment of inflammation status of BAT. Collagen fibers were stained by Van Gieson trichrome with hematoxylin as a counterstain [27]. Quantitative determination of tissue collagen content was performed on digital microphotographs. All captures were obtained using a light microscope BX41 (Olympus, Japan) with 4× objective lens. Microphotographs were taken with DP20 digital camera (Olympus, Japan) and QuickPHOTO

MICRO software (Promicra, Czech Republic). The field of view of each subsequent microphotograph was moved to the right, retaining 20 % of the previous field until the entire specimen was taken. To create one large composite panoramic image, the cross-linking software Adobe Photoshop CS6 (Adobe Systems, USA) was used. Area of the red-stained region, which corresponds to collagen fibers was calculated and expressed as a percentage of the total area [28].

Histological evaluation of the inflammation level in the BAT was performed according to the generally accepted semi-quantitative score scale of leukocyte infiltration: absence – 0, insignificant – 1, medium – 2, noticeable – 3 [29]. We investigated at least 5 similar areas on each section. All parameters were measured with ImageJ software (National Institutes of Health, USA).

Statistical data analysis was performed using the Statistica 6.0 (StatSoft, USA) and Microsoft Excel 2010 software (Microsoft, USA). The obtained data was presented as mean ± standard error of mean (SEM). The distribution of data was assessed with Shapiro-Wilk normality W-test. Since the analyzed distributions were considered normal, we used one way ANOVA followed by Dunnett's multiple range post-hoc test to evaluate the differences between means. The differences with probability of the null hypothesis $p < 0,05$ were considered significant.

Results and discussion. Brown adipocytes in control group showed typical morphology: polygonal shape, rounded nuclei that occupy a central position in cell and many tiny-sized lipid inclusions in cytoplasm (Fig. 1). Large collagen deposits were localized in parts of capsule and trabeculae of BAT. Collagen fibers were mostly present around blood vessels. We did not find any significant collagen accumulations between brown adipocytes. Similarly, substantial collagen deposits were absent near white adipocytes, which surround BAT and continuously underlie its peripheral part. Development of obesity was accompanied by the change of brown adipocytes' morphology: round shape of the cells, flat nucleus in eccentric position and unilocular massive lipid droplet, which corresponds to a white adipose tissue appearance [30]. Collagen deposits in HCD group expanded markedly in interlobular zone and near blood vessels (Fig. 1, red arrow). We detected collagen as zones of red staining, while yellow coloring indicated little to no collagen content. Intriguing, BAT collagen fibers of obese rats were closely located to some brown adipocytes, to the majority of "whitening" brown adipocytes and to many peripherally located white adipocytes. In addition to enlarged collagen fibers, in HCD group we detected presence of leukocytes (macrophages and lymphocytes) in BAT (Fig. 1, arrowheads).

Melatonin administration (M ZT01 and M ZT11 group) did not affect BAT collagen content in both trabecular and intercellular spaces of rats which received standard diet (Fig. 2). Also, we did not detect visible tissue leukocyte infiltration. We have previously described the most noticeable effects of melatonin application on morpho-functional state of brown adipocytes in rats, consumed standard diet [31]. It was mainly manifested in increased number of lipid droplets per adipocyte, cross-section area of the nucleus of brown adipocytes (and, respectively, nuclear-cytoplasmic ratio of brown adipocytes was also elevated), optical density of BAT, whereas cross-sectional area of lipid droplets in brown adipocytes decreased significantly.

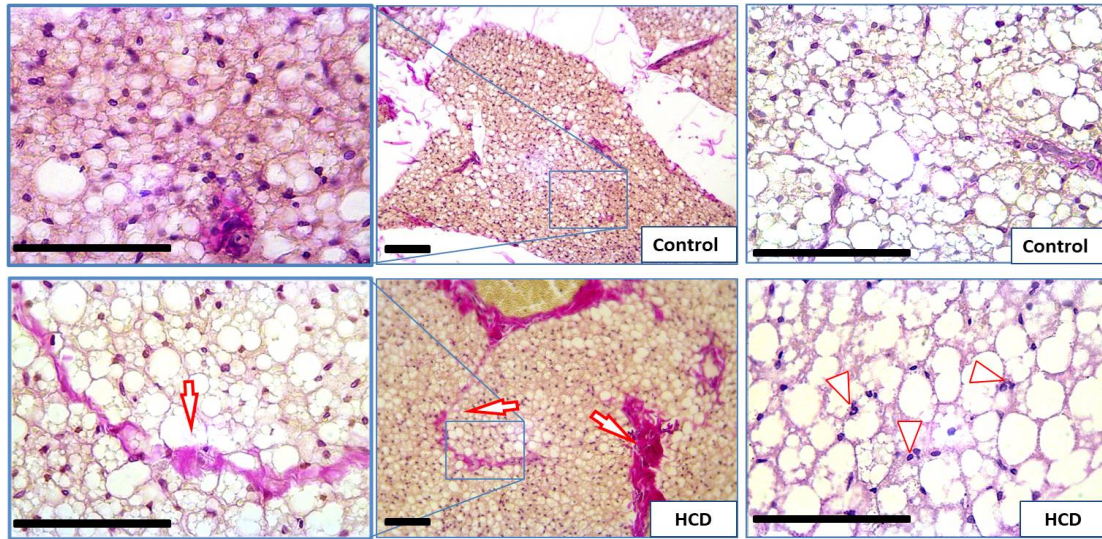


Fig. 1. Microphotographs of rats' brown adipose tissue sections: Van Gieson trichrome staining; Control and HCD groups; scale bar: 100 μm

Notes. Red arrow – collagen deposits (red color), arrowheads – leukocyte infiltration.

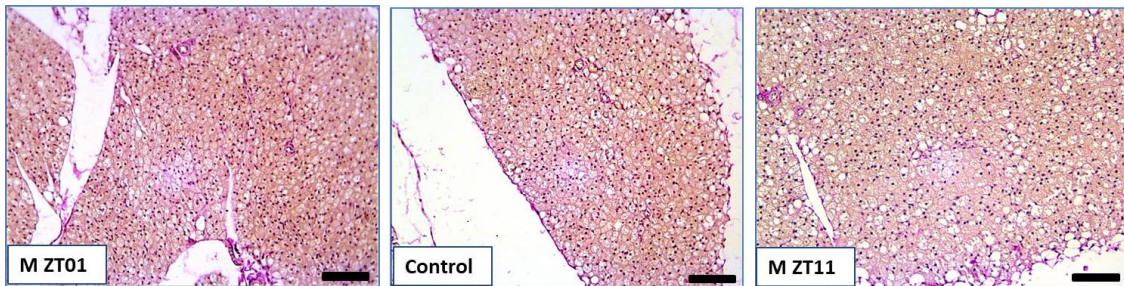


Fig. 2. Microphotographs of rats' brown adipose tissue sections: Van Gieson trichrome staining; Control and M ZT01, M ZT11 groups; scale bar: 100 μm

Distribution of collagen fibers in groups HCD ZT01 and HCD ZT11 (Fig. 3) was different from control group (red arrows) – it was elevated in interlobular zone and near blood vessels, but still remarkably smaller than in HCD.

However, we did not observe massive collagen fibers that surround each individual adipocyte as in central and in peripheral zones of BAT. In addition, rare clusters spreading of leukocytes infiltration in BAT were detected.

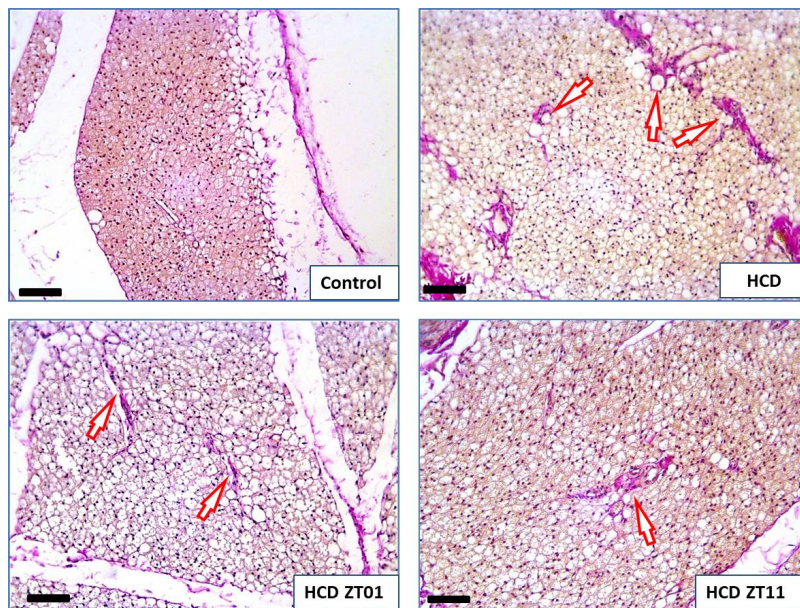


Fig. 3. Microphotographs of rats' brown adipose tissue sections: Van Gieson trichrome staining; Control, HCD, HCD ZT01 and HCD ZT11 groups; scale bar: 100 μm

Notes. Red arrow – collagen deposits (red color).

Morphometric analysis of obtained data demonstrated significant 2-fold increase in BAT fibrosis level of obese rats after consuming high-calorie diet comparing to control (Fig. 4). Melatonin administration during HCD-induced obesity prevented the increase of BAT collagen content after both morning and evening interventions: fibrosis level in HCD ZT01 group decreased by 56 % and by 44 % in HCD ZT11 group in comparison to HCD group. The fibrosis levels in HCD ZT01 and HCD ZT11 groups did not differ from control value. There was no significant difference between morning and evening regimes of melatonin administration in HCD ZT11 and HCD ZT01 groups in tissue collagen content. Also variations in melatonin regimes did not influence on BAT fibrosis development in rats consuming standard diet.

HCD-induced development of obesity resulted in significant 5-fold tissue leukocyte infiltration, comparing to control group (Fig. 4). As obesity is usually accompanied by low-grade chronic pro-inflammation state, the increased

tissue leukocyte infiltration was also detected in HCD ZT01 and HCD ZT11 groups after melatonin administration (3,4-fold in HCD ZT01 and 2,2-fold in HCD ZT11 groups). However, this parameter was significantly lower than in HCD group (HCD ZT01 – 1,5 times, HCD ZT11 – 2,3 times). Additionally, we detected significant difference between morning and evening regimes of melatonin administration in tissue leukocyte infiltration levels: it was lower by 55% in HCD ZT11, comparing to HCD ZT01. Melatonin treatment in morning or evening regimes did not influence on BAT tissue leukocyte infiltration in groups consuming standard diet.

Taking into account the results of morphological observations and morphometric analysis, melatonin was shown to have the corrective effect of on the BAT inflammation state during obesity development without any destructive influence on BAT of rats, consuming standard diet.

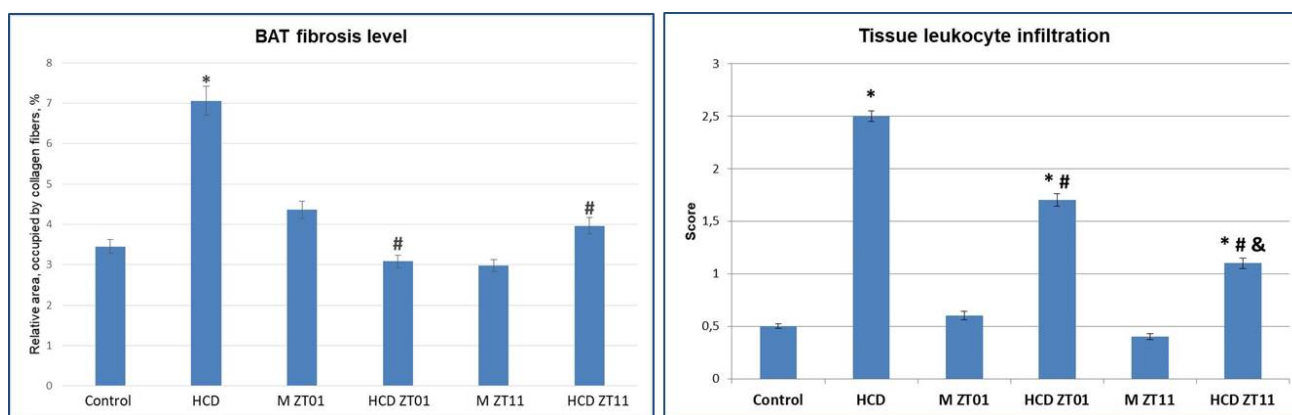


Fig. 4. Brown adipose tissue fibrosis level and tissue leukocyte infiltration

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 $p \leq 0,05$; & – difference between the HCD ZT01 and HCD ZT11 groups is significant at $p \leq 0,05$.

Our data generally correspond to previously demonstrated increase in brown adipose tissue mass and functional activity in Zucker diabetic fatty rats. These animals were treated for 6 weeks with melatonin in drinking water in the dose of 10 mg/kg body weight. The consumption of melatonin took place mainly at night as rats are nocturnal animals [32]. Unfortunately, there is no similar study with investigation of melatonin effects on BAT fibrosis under HCD-induced obesity. It may be related to a work that showed lower BAT fibrosis levels comparing to white adipose tissue, which was demonstrated on high-fat diet-induced obesity in C57BL6/J mice model [33]. A huge research limitation associated with the use of C57BL6/J mice is that this strain is notoriously known for being deficient in the biosynthesis of melatonin [34] and short-term lasting obesity model. Another study on diet-induced obesity showed significant alterations of BAT molecular networks associated with immune cell trafficking, lipid metabolism and connective tissue development after 24 weeks of diet consumption [35]. High fat diet-induced obesity resulted in reduction of UCP1 levels and overexpression of pro-inflammatory genes; while aerobic exercise led to the growth of BAT and the upregulation of anti-inflammatory genes in obese mice via cyclooxygenase 2 in the VEGF pathway [36]. Another model of diet-induced obesity in rats established development of BAT fibrosis and modulation of this parameter by intermittent food restriction [37].

Conclusions. Daily administration of exogenous melatonin (30 mg/kg, 7 weeks) in different regimes

demonstrated the corrective effect on the BAT inflammation status during high-calorie diet-induced obesity, without any pathological influence on BAT of rats that consumed standard diet. The fibrosis levels following melatonin usage in rats with obesity generally corresponded to control values, while the tissue leukocyte infiltration occupied intermediate position: infiltration was lower in comparison to obese rats, but also higher than in control. Therefore, different modes of 30 mg/kg exogenous melatonin applications to obese rats for 7 weeks led to decreased manifestation of low-grade brown adipose tissue inflammation through reduced fibrosis and tissue leukocyte infiltration.

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ОСОБЛИВОСТІ ЗАПАЛЕННЯ БУРОЇ ЖИРОВОЇ ТКАНИНИ У ЩУРІВ

З РОЗВИТКОМ ІНДУКОВАНОГО ДІЄТОЮ ОЖИРІННЯ ПІСЛЯ РІЗНИХ РЕЖИМІВ УВЕДЕННЯ МЕЛАТОНІНУ

Однією із характерних рис ожиріння є розвиток системного хронічного прозапального стану. Буря жирова тканина (БЖТ) може бути потенційною мішенню для активування мелатоніном виробництва тепла і, водночас, стимуляції ліполізу під час розвитку ожиріння. Також мелатонін має імуномодуляторні властивості, що реалізуються за участі ендокринних і паракринних сигнальних шляхів у різних типах клітин (зокрема і в бурих адипоцитах) і значно варіюють упродовж доби. Це уможливило його застосування в широкому діапазоні доз і режимів уведення (хронотерапевтичний підхід). Отже, основна мета нашого дослідження – проведення аналізу стану запалення БЖТ у щурів за розвитку ожиріння, індукованого висококалорійною дієтою, після щоденного введення мелатоніну в різний час (уранці й увечері). Мелатонін уводили інтрагастралью через зонд протягом 7 тижнів в дозі 30 мг/кг за 1 год до вимкнення світла (ВКД ZT11, M ZT11, вечір) або через 1 год після увімкнення світла (ВКД ZT01, M ZT01, ранок). Для оцінювання стану запалення (умісту колагену у тканинах та рівня інфільтрації лейкоцитами) у БЖТ використовували гістохімічне фарбування трихромом за ван Гізеном. За розвитку ожиріння рівень фіброзу, який оцінювали за відносною площею, що займають колагенові волокна, й інфільтрація лейкоцитами у БЖТ збільшувалися порівняно зі щурами контрольної групи. Після застосування мелатоніну рівень фіброзу у щурів з ожирінням у груп ВКД ZT01 і ВКД ZT11 не відрізнявся від контрольних значень. Крім того, подібний ефект було виявлено у процесі аналізу інфільтрації лейкоцитами БЖТ щурів з ожирінням за обох режимів уведення мелатоніну (групи ВКД ZT01 і ВКД ZT11): цей параметр був досто-

вірно нижчим, ніж у щурів з індукованим ожирінням, але, водночас, більшим порівняно зі щурами, які не страждали на ожиріння. Також уведення мелатоніну в ранковому або вечірньому режимах не впливало на стан сполучної тканини БЖТ щурів, що еживали стандартну дієту (групи M ZT01 і M ZT11). Загалом дослідження показує корисувальні властивості мелатоніну в умовах хронічного запалення у БЖТ щурів з ожирінням і передбачає широкий потенціал використання мелатоніну за відсутності побічного ефекту на гістофізіологію БЖТ у щурів без ожиріння.

Ключові слова: хронобіологія, гістохімія, бурі адипоцити, фіброз, лейкоцитарна інфільтрація, індуковане висококалорійною дієтою ожиріння.

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ОСОБЕННОСТИ ВОСПАЛЕНИЯ БУРОЙ ЖИРОВОЙ ТКАНИ КРЫС С РАЗВИТИЕМ ИНДУЦИРОВАННОГО ДИЕТОЙ ОЖИРЕНИЯ ПОСЛЕ РАЗНЫХ РЕЖИМОВ ВВЕДЕНИЯ МЕЛАТОНИНА

Одной из характерных черт ожирения является развитие системного хронического провоспалительного состояния. Бурая жировая ткань (БЖТ) может быть потенциальной мишенью для активации мелатонином производства тепла и, в то же время, стимуляции липолиза во время развития ожирения. Также мелатонин обладает иммуномодулирующими свойствами, которые осуществляются эндокринным и паракринным сигнальными путями в различных типах клеток (включая бурые адипоциты) и значительно изменяются в течении суток, поэтому его применение возможно в широком диапазоне доз и в разное время суток (хронотерапевтический подход). Таким образом, основной целью нашего исследования было проведение анализа состояния воспаления БЖТ у крыс при развитии ожирения, индуцированного высококалорийной диетой, после ежедневного введения мелатонина в разное время (утром и вечером). Мелатонин вводили интрагастрально через зонд в течение 7 недель в дозе 30 мг/кг за 1 час до выключения света (ВКД ZT11, M ZT11, вечер) или через 1 час после включения света (ВКД ZT01, M ZT01, утро). Для оценки состояния воспаления (содержания коллагена в тканях и степени инфильтрации лейкоцитов) в БЖТ использовали гистохимическое окрашивание трихромом по ван Гизону. При развитии ожирения уровень фиброза, который оценивали по относительной площади, занимаемой коллагеновыми волокнами, и инфильтрация тканевых лейкоцитов в БЖТ увеличивались по сравнению с крысами контрольной группы. После применения мелатонина уровень фиброза у крыс с ожирением групп ВКД ZT01 и ВКД ZT11 не отличался от контроля. Кроме того, подобный эффект наблюдался при анализе инфильтрации лейкоцитами БЖТ крыс с ожирением за обоих режимов введения мелатонина (группы ВКД ZT01 и ВКД ZT11): этот параметр был значительно ниже, чем у крыс с ожирением, но все же увеличился по сравнению с крысами, не страдающими на ожирение. Также введение мелатонина в утреннем или вечернем режимах не влияло на развитие фиброза и инфильтрацию лейкоцитами в БЖТ крыс, употребляющих стандартную диету (группы M ZT01 и M ZT11). В целом, исследование показывает корректные свойства мелатонина в условиях хронического воспаления в БЖТ крыс с ожирением и предусматривает широкий потенциал использования мелатонина при отсутствии побочного эффекта на гистофизиологию БЖТ у крыс без ожирения.

Ключевые слова: хронобиология, гистохимия, бурые адипоциты, фиброз, лейкоцитарная инфильтрация, индуцированное высококалорийной диетой ожирение.