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EFFECT OF LONG-TERM CONSTANT DARKNESS ON RETINOL IN PERIPHERAL TISSUES OF RATS

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The study was designed to investigate the effect of exposure to long-term constant darkness, starting from the prenatal period or from birth, on the retinol (vitamin A) content in tissues of rats. Females were kept in standard light (LD) or in constant darkness (DD) during pregnancy. The LD females and their offspring after birth were divided into two groups, one of which was left in the same lighting conditions (LD, control), and the other group was switched to the darkness regime (LD/DD). The DD females and their offspring (DD/DD) were kept in the dark. Adults and the young were separated after the suckling period. The retinol content in offspring's tissues (liver, kidneys, heart and skeletal muscle) was determined at the age of 2 weeks, 1, 2, 3, 6, 12, 18 and 24 months by HPLC. Constant darkness had a modulating effect on the age-related dynamics of retinol content in the tissues. The level of the vitamin was higher compared to the control animals in the liver of 2-week-old LD/DD rats and in the kidneys of 1-month-old rats of both experimental groups. The retinol content in the heart of 2-month-old DD/DD rats, on the contrary, was significantly lower than in the control. The retinol level in the liver of 12-month-old LD/DD rats was higher compared to the control animals. The effect of constant darkness on retinol level in tissues depended on the ontogenesis stage at which the experimental exposure began, the tissue type, and the animal's age. The retinol content in the tissues of rats kept in constant darkness indicates metabolic changes that were more pronounced in young animals.

Keywords: vitamin A; constant darkness; light; circadian rhythm; age

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Т. Н. Ильина, И. В. Баишникова*, Е. А. Хижкин. ДЛИТЕЛЬНОЕ ВЛИЯНИЕ ПОСТОЯННОЙ ТЕМНОТЫ НА СОДЕРЖАНИЕ РЕТИНОЛА В ПЕРИФЕРИЧЕСКИХ ТКАНЯХ КРЫС

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Целью работы было изучение влияния длительной постоянной темноты, начиная с внутриутробного периода или с момента рождения, на содержание ретинола

(витамин А) в тканях крыс. Самки во время беременности содержались при стандартном освещении (LD) или в постоянной темноте (DD). Самки LD и их потомство сразу после рождения были разделены на две группы, одну из которых оставили в тех же условиях (LD, контроль), а другую перевели в темноту (LD/DD). Самки DD и их потомство (DD/DD) находились в темноте. Взрослые особи и молодняк были разделены после подсосного периода. Содержание ретинола в тканях (печень, почки, сердце, скелетная мышца) определяли в возрасте 2 недель, 1, 2, 3, 6, 12, 18 и 24 месяцев методом ВЭЖХ. Постоянная темнота оказывала модулирующее влияние на возрастную динамику содержания ретинола в тканях. Уровень витамина был выше по сравнению с контрольными животными в печени 2-недельных крыс LD/DD и в почках 1-месячных крыс обеих опытных групп. В сердце 2-месячных крыс DD/DD содержание ретинола, напротив, было достоверно ниже, чем у контрольных. В печени 12-месячных крыс LD/DD уровень ретинола был выше, чем у контрольных животных. Влияние постоянной темноты на уровень ретинола в тканях зависело от стадии онтогенеза, на которой начиналось экспериментальное воздействие, типа ткани и возраста животных. Содержание ретинола в тканях крыс, находившихся в постоянной темноте, свидетельствует о метаболических изменениях, которые были более выражены у молодых животных.

Ключевые слова: витамин А; постоянная темнота; свет; циркадный ритм; возраст

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Финансирование. Финансовое обеспечение исследований осуществлялось из средств федерального бюджета на выполнение государственного задания (тема FMEN-2022-0003).

Introduction

Physiological processes are inextricably linked with cyclical changes in the general level of vital activity. The main synchronizer of the organism's circadian system is the alternation of light and darkness. The mammalian central circadian clock is located in the hypothalamic suprachiasmatic nucleus (SCN), however, circadian clocks also exist in peripheral tissues, which indicates the close relationship between circadian regulation and metabolism [Ko, Takahashi, 2006; Grimaldi, Sassone-Corsi, 2007; Lamia et al., 2008; Chang et al., 2016]. Internal circadian rhythms are subject to the change of day and night cycles, and the connection of the organism with the external environment begins to form at the prenatal stage. Constant darkness, as well as constant light, is considered as a form of ecological stress that raises the risk of metabolic diseases [Ruby et al., 2002; Lee, 2007; Yuksel, 2008; Panda, 2016]. In the conditions of constant darkness, the circadian clock freely runs with its own period, different from but close to 24 h. The light-dark cycle plays a key role in determining the level and duration of secretion of the pineal hormone melatonin, which is synthesized in the dark and whose main function is the regulation of biological rhythms. In addition to regulating biorhythms, endogenous melatonin acts as an antioxidant and the main directions of its antioxidant action are to protect the cell macromolecules from oxidative damage and increase the antioxidants' efficiency [Reiter, 2000; Chitimus et al., 2020]. It is well known that seasonal alterations of natural daylight duration in high or moderate latitudes trigger numerous adaptive changes in wild animals. Decrease in natural illumination during autumn/winter months can cause seasonal affective disorders characterized by overeating, weight gain, hypersomnia, prominent fatigue, and some other changes in people. Seasonal disorders related to the change in illumination are observed in 11–21 % of individuals and are a social and economic problem [Bazhenova et al., 2019].

Vitamin A (retinol, VA) participates in many physiological functions including vision, embryonic development, growth, reproduction, cell differentiation and proliferation, and together with its derivatives can act as an antioxidant [Estornell et al., 2000; Gatica et al., 2012]. One of the main VA functions is the control of biological rhythms. Retinoic acid (RA), the bioactive form of VA, is reported to affect the circadian rhythm by binding to RA receptors, such as receptors in the circadian feedback loops in the mammalian SCN [Guo et al., 2022]. It was found that VA is necessary for the functioning of the pineal gland, which contains a high level of retinol and retinol-binding protein. A deficiency of VA leads to a decrease in the melatonin night peak

[Phillips et al., 1989; Ransom et al., 2014; Ashton et al., 2015]. Moreover, its deficiency may affect the photo-response, sleep cycle, cell metabolism, and induce disorders/diseases related to biological rhythm dysfunctions [Guo et al., 2022]. At the same time, it remains unclear how the light-dark cycle disturbance affects the content of vitamin A in the body. The purpose of this work was to investigate the long-term effect of constant darkness, starting from the prenatal period or from birth, as a model of light rhythm disturbance, on the retinol content in rat tissues.

Materials and methods

Objects and experimental design

This study was carried out in compliance with the Directive 2010/63/EU of the European Parliament and of the Council (On the Protection of Animals used for Scientific Purposes, 2010) and approved by the Local Ethics Committee of the Institute of Biology of the Karelian Research Centre of the Russian Academy of Sciences (Approval No 10 dated October 3, 2016). Every effort was made to minimize as much as possible the number of animals and their suffering. The experiment was performed on Wistar rats kept under standard vivarium conditions with free access to rodent chow and water. For breeding, 4-month-old male and female rats were kept under standard fixed lighting conditions (12 h light 750 lux/12 h darkness; LD) or in constant darkness (0–0.5 lux; DD). All DD rat manipulations were performed under dim red light within 3 min. After delivery, females from the LD conditions and their offspring were randomly divided into two groups and either remained housed in the same lighting conditions (LD, control) or transferred to constant darkness (LD/DD). Females and their offspring from the DD lighting conditions were left in constant darkness (DD/DD). After weaning at three weeks of age, the offspring were separated by gender and kept in the same type of cages (4 rats per cage) until 24 months of age. Animals of all groups received the same and age-appropriate food. The rats from each group (n = 8) were decapitated at the same time in the morning after light diethyl ether anesthesia at the age of 2 weeks and 1, 2, 3, 6, 12, 18 and 24 months. Samples of the liver, kidneys, heart, and skeletal muscles were collected and stored at –80 °C until analysis. All animals used for the study were in good health and showed no signs of illness.

Retinol determination

The retinol content in the tissues was determined by high-performance liquid chromatography (HPLH) [Skurihin, Dvinskaya, 1989]. Tissue samples (100 mg) were homogenized with a teflon pestle homogenizer in 0.9 ml of 0.25 M sucrose solution (pH 7.4). Proteins in the samples were precipitated by ethanol. Retinol was extracted by n-hexane. The ethanol and hexane used for extraction contained butylated hydroxytoluene to prevent the vitamin oxidation during the analytical procedure. The mixture was vortexed for 5 min to extract the vitamin, centrifuged at 3000×g for 10 min, and kept for 40 min at 4 °C. Chromatographic separation was carried out by a microcolumn chromatograph with a UV detector. Hexane and isopropanol mixture (98.5 : 1.5) was used as the eluent. Retinol concentration was determined in the hexane layer. The sample volume injected into the column was 10 µl. The eluate was monitored at 324 nm. The retinol was identified by comparison with the retention time of pure standard (Sigma-Aldrich, USA). Quantification was performed using Uni-Chrom software by the external standard method. The research was carried out using the equipment of the Core Facility of the Karelian Research Centre RAS.

Statistical analysis

All the calculated numerical data were converted to SI units and expressed as the median and percentiles (25%, 75%). The results were processed using Microsoft Excel 2007 and Statgraphics 5.0 software using Kruskal–Wallis H-test with the Mann–Whitney U *post hoc* testing as appropriate (adjusted for multiple comparisons). Differences were considered statistically significant at a significance level below 0.05. Preliminary tests revealed no differences between sexes, therefore data for males and females were pooled together in all subsequent analyses.

Results

The dynamics of changes in the content of retinol in liver was similar in rats of all groups up to 12 months of age: until the age of one month, the level of the vitamin was low, and then it increased up to 3 months ($p < 0.05$, Fig. 1, a). Until 12 months of age, the content of retinol continued to increase more intensively in rats of the experimental groups, but no significant differences were found in the level of the vitamin at 3 months of age. In the LD/DD group, vitamin levels were significantly lower at 18 months of age than at 6 and 12 months ($p < 0.05$). In LD and DD/DD rats, the patterns were similar, but not significant. At the age of 24 months, the content of retinol increased, and in the LD and LD/DD groups it was significantly higher than at the previous age ($p < 0.05$). Although the retinol

content in tissues in the early postnatal period was low, the effect of lighting conditions was revealed even in this period. At 2 weeks of age, the level of retinol in rats of the LD/DD group was significantly higher compared to the animals of the control and the DD/DD groups ($p < 0.05$). The same difference between LD and LD/DD groups was revealed in 12-month-old rats ($p < 0.05$).

In the kidneys of control rats, the content of retinol significantly decreased at 1 month of age compared with 2 weeks of age ($p < 0.05$, Fig. 1, b). Then, by the age of 2 months, the level of the vitamin increased significantly ($p < 0.05$) and then changed slightly during the rest of the study period. In both experimental groups, the content of retinol increased up to 6 months of age, the change in LD/DD rats being statistically significant compared to all previous ages, and in DD/DD rats only compared with 2 weeks and 1 month of age. At 12 months, the vitamin level decreased significantly: in the LD/DD group it was lower compared to the previous age and in the DD/DD group – compared to 2 months of age. Subsequent changes in the content of retinol in experimental groups, as well as in the control group, were insignificant. The influence of lighting conditions on the level of retinol in kidneys was revealed at 1 month of age: the vitamin content was significantly higher in rats kept in constant darkness than in the control group ($p < 0.05$).

In the heart of LD rats, the level of retinol increased significantly at 2 months of age, after

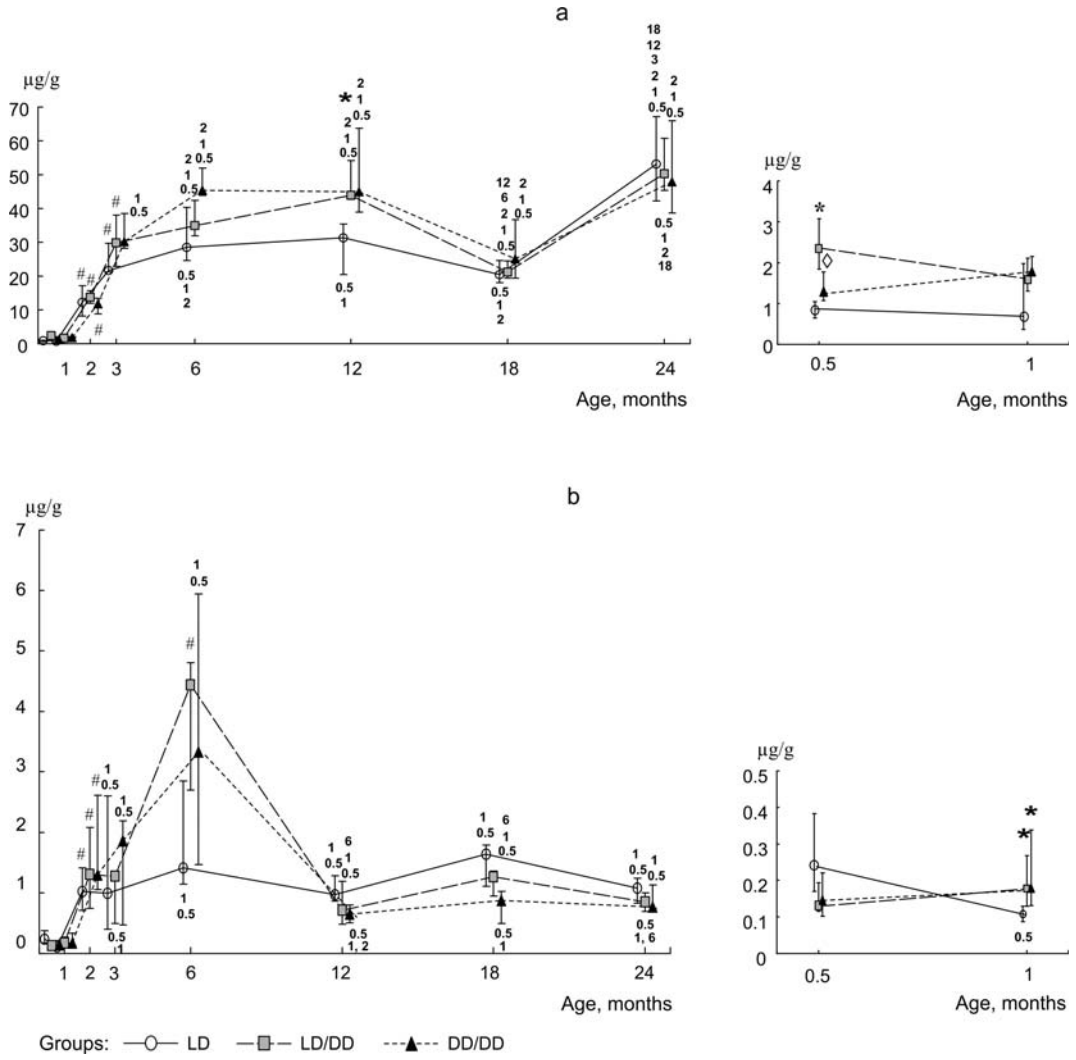

Fig. 1. The retinol content in the liver (a) and kidneys (b) of rats kept under different lighting conditions.

Designations: the graphs on the right show changes in retinol content in rats aged from 0.5 to 1 month. Here and in Fig. 2, differences are significant compared to: * – the LD group; ◊ – the LD/DD group; 0.5, 1, 2, 3, 6, 12, 18 – the corresponding age; # – all previous ages (Mann–Whitney U-test, $p < 0.05$). The values are represented as median and percentiles (25%, 75%)

which the content of this vitamin declined until 6 months of age ($p < 0.05$, Fig. 2, a). Subsequently, the vitamin level gradually increased and it was significantly higher in 24-month-old rats than in 6-month-old ones ($p < 0.05$). In animals of the experimental groups, the level of retinol rose after 1 month of age, the highest values being recorded in 24-month-old rats ($p < 0.05$). At 2 months of age, the content of retinol in the heart of DD/DD rats was significantly lower than in the control animals ($p < 0.05$).

In the skeletal muscle of control rats, the level of retinol increased at an older age and was higher in 18- and 24-month-old animals than in those 3 months old ($p < 0.05$, Fig. 2, b). In the LD/DD group, the vitamin content in the oldest rats (24 months old) was higher than in 12- and 18-month-old animals ($p < 0.05$). In 6-month-old DD/DD rats, a significant decrease in retinol content was observed compared to 3-month-old rats ($p < 0.05$), after which the vitamin level increased and reached the highest values in 24-month-old animals ($p < 0.05$). Lighting conditions did not significantly affect the content of vitamin A in the skeletal muscle.

The studied animals grew most intensively until 2 months of age (Fig. 3). After that, the body weight continued to increase, the difference versus 2-month-old rats found in 12-, 18- and 24-month-old LD rats and in 18-and 24-month-old DD/DD rats. In the LD/DD group, body weight at 12, 18 and 24 months of age differed significantly also from that at 3 months. Long-term exposure to constant darkness had no significant effect on the body weight dynamics of rats.

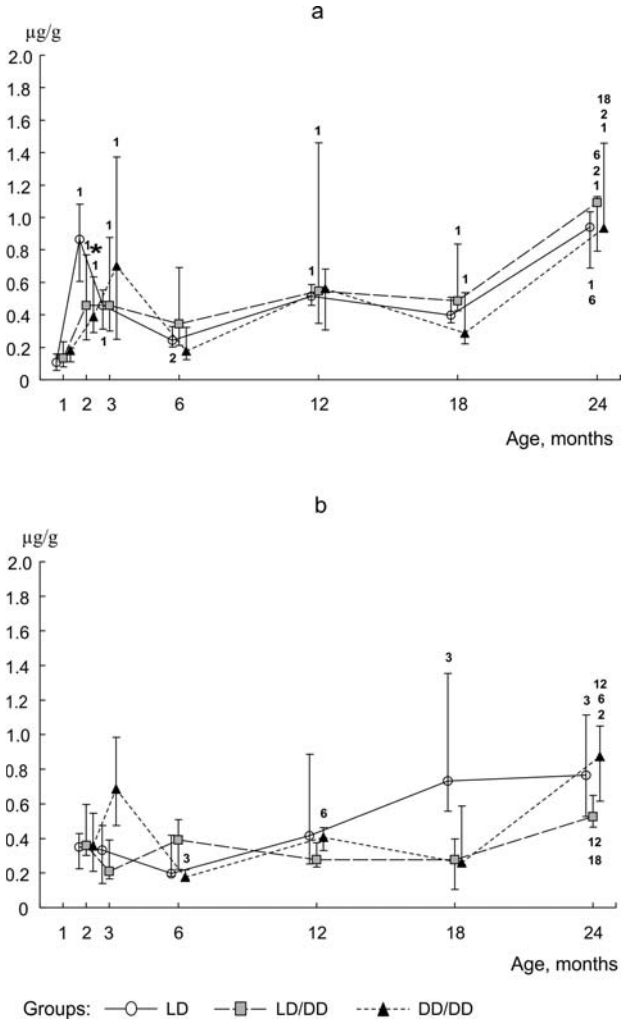

Fig. 2. The retinol content in the heart (a) and skeletal muscle (b) of rats kept under different lighting conditions

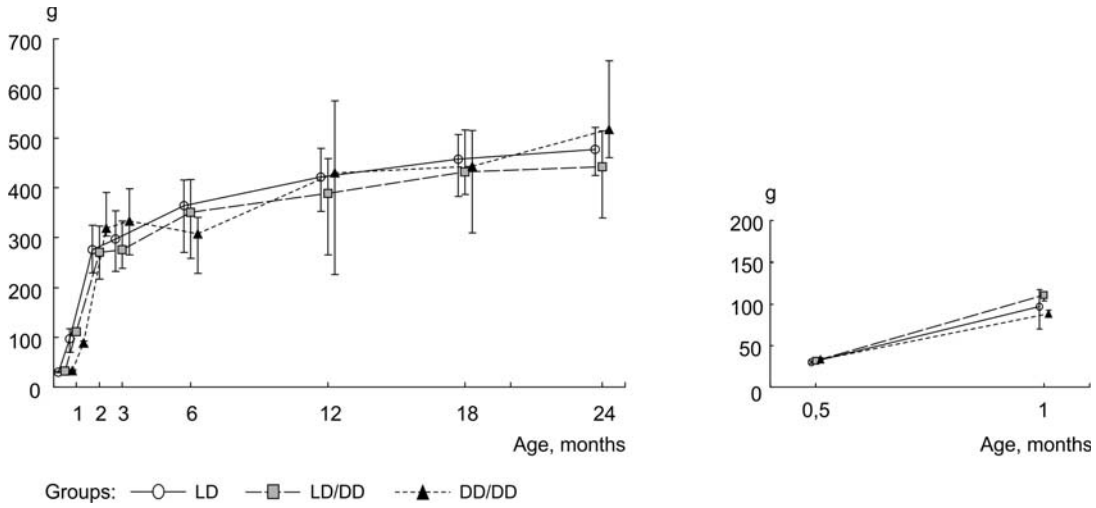

Fig. 3. Body mass of rats kept under different lighting conditions.

Designations: the graph on the right shows changes in body mass of rats aged from 0.5 to 1 month. Differences are significant compared to: 0.5, 2, 3 – the corresponding age; & – ages 0.5 and 1 month (Mann–Whitney U-test, $p < 0.05$). The values are represented as median and percentiles (25%, 75%)

Discussion

The connection of the organism with the external environment in mammals is established already in the prenatal period through maternal endogenous signals that can influence the trajectory of postnatal development. Information about the photoperiod and its alterations is transmitted to a fetus via the maternal melatonin rhythm. The maturation of an own circadian system in rodents occurs only after birth. It was revealed that retinol metabolites affect clock genes and the formation of the circadian rhythm. Disturbances of the lighting conditions before the end of the circadian clock mechanism formation may have negative consequences for the metabolic and behavioral functions of offspring [Bishnupuri, Haldar, 2000; Chernysheva et al., 2012; Chitimus et al., 2020].

Retinol levels in new-born mammals are very low, which is due to poor permeability of the placenta for fat-soluble vitamins. At the same time, retinol recirculation in newborns occurs much more intensively than in adults, which contributes to the rapid delivery and accumulation of VA in tissues after birth [Tan et al., 2014, 2017]. Our study detected low retinol content in tissues in the early postnatal period, which increased significantly with age. On the other hand, already in the early ontogenesis significant differences were found in the hepatic and renal VA content between control and experimental rats, which were obviously caused by the lack of photoperiodicity in the rat females during pregnancy or after delivery.

The action of retinol is mediated by its metabolite retinoic acid, which is also produced in melatonin-synthesizing pinealocytes. It is obvious that melatonin and VA, as components of the circadian clock, do not work separately, but are parts of a single system [Herbert, Reiter, 1985; Ransom et al., 2014; Ashton et al., 2018]. The combined action of changes in various components of the VA metabolism has a significant effect on the retinol level in tissues. It has been shown that there is diurnal variation in plasma levels of retinol binding protein 4 in mice and also in the expression of the gene encoding cellular retinol binding protein 1 in the liver, which peaks during subjective night [Ashton et al., 2018]. Thus, an increase in the retinoic acid concentration at the end of the night compared with its beginning, as well as an increase in the level of retinol transport proteins, may contribute to increased night VA absorption. The action of retinoic acid in the pineal gland is controlled by two modes of regulation, one of which is orchestrated by the endogenous circadian clock, and the other one depends on changes in the light-dark cycle [Akashi, Takumi, 2005; Ashton et al., 2018].

In contrast to the light-regulated SCN clock, peripheral clocks in tissues that are not directly affected by light are set by daily nutrition, contributing to metabolic regulation. The close relationship between circadian and metabolic cycles is supported by the nutrition rhythm influence on the clock phase in many peripheral tissues, including the liver, the kidneys, the heart and the skeletal muscles [Turek et al., 2005; Asher, Schibler, 2011; Gnocchi et al., 2015; Chang et al., 2016]. Being nocturnal animals, rats prefer to consume food during the active dark period, even if they are raised in a light-dark cycle and in *ad libitum* feeding conditions. Circadian rhythms and gene functions force the body to consume nutrients even in the absence of light signals [Ko, Takahashi, 2006; Panda, 2016]. Probably, keeping rats in the dark promotes the retinol content in tissues, and this first of all applies to the liver as the main depositing organ. The VA content in other tissues depends on the liver circadian rhythmicity, however, the central clock photoperiodic modulation and the peripheral clock in the liver may differ significantly [Hirao et al., 2006; Sosniyenko et al., 2010; Gnocchi et al., 2015]. Our study showed that the transfer of rats to the constant darkness conditions after birth affected the retinol level in the liver. The circadian clock mechanism forming in the offspring adapts to the “disconnection” from the mother’s metabolic clock and “switching over” to the circadian light variation cycles [Chernysheva et al., 2012]. Changing the lighting condition immediately after birth was of great importance for the retinol level, since significant differences were observed in comparison with the control as well as with DD/DD rats. This may indicate a change in the level of VA under the impact of circadian factors in the liver rather than directly in the SCN, whose circadian regulation is disrupted. Differences from the control group were found in LD/DD rats also at 12 months of age. It should be noted that a higher content of retinol in the liver was observed in both groups of rats kept in constant darkness from the age of 3 months to the age of one year. The response to the light rhythm disruption varies with age. No effect of the lighting conditions on VA was detected in the liver of old rats, which may indicate age-related changes in the circadian system.

Various functions demonstrate circadian rhythms in rodent kidneys, with most renal functional oscillations entrained by external circadian time cues [Hara et al., 2017; Firsov, Bonny, 2018]. Disruption of circadian rhythms can affect various cellular processes, including protein pathways. It

is known that an important role in the regulation of VA homeostasis in the body is played by megalin-mediated reuptake of retinol and retinol-binding protein in the kidneys [Raila et al., 2005]. Exposure to constant darkness in the prenatal and postnatal periods led to distinct shifts in the rats' renal retinol level. The content of retinol in kidneys of animals of the experimental groups was higher than in the control at the age of 1 month. After this age, the vitamin level increased in all groups, but the most substantial and statistically significant increase up to 6 months of age was observed in the LD/DD group. In one-year-old rats of this group, the content of retinol in kidneys decreased and was comparable to that in the control and the DD/DD group. The peripheral clock regulation is complex and includes many additional components that affect the physiological parameters that are associated with SCN, which plays a major role in clock adjustment in the peripheral organs, including the liver and kidneys. Circadian oscillators in the brain, muscles and internal organs are interconnected, but at the same time differ in time parameters and control systems due to the peculiarities of their metabolism [Lamia et al., 2008]. Thus, mice with SCN damage maintained regular periodicity in the liver and kidneys, but not in skeletal muscles and heart [Gnocchi et al., 2015], which emphasizes the complexity of the circadian system and the interaction between various regulatory mechanisms. Our study showed that changes in vitamin A levels in organs indicate different sensitivity of peripheral tissues to circadian rhythm disturbances.

The specific contribution of each component of the molecular clock to circadian rhythms regulation is tissue-specific. It has been shown that in adult rodents circadian variations in cardiac and skeletal muscles depend, among other things, on the type of muscle tissue. Circadian regulation in different types of muscles depends on the fibers composition, tissue metabolism and the level of its activity, which is determined by various tissue-specific functions. Changes in lighting and nutrition can significantly affect the balance of muscle proteins, which is closely related to the VA metabolism [Chang et al., 2016]. We found that in the heart, the content of retinol increased by 2 months of age in all groups, but most substantially in the control. At this age, the vitamin level in the control rats was significantly higher than in the DD/DD group. Since the heart is a retinoid-dependent organ [Asson-Batres et al., 2016], this increase could be associated with age-related changes in energy processes in the myocardium, as well as with changes in the VA level in the body. The lower content of retinol in the heart of rats kept in the dark could be caused by changes in the pathway of retinoid enzymes, binding proteins, and transporters under circadian rhythm disturbance.

The skeletal muscle is one of the most important organs for storing the substrates necessary for the body [Dyar et al., 2015; Chang et al., 2016; Aisbett et al., 2017]. There is a circadian difference in muscle growth during day and night – the growth of muscle tissue is about twice as much during the day as at night [Dyar et al., 2015], therefore exposure to constant light or constant darkness leads to changes in the circadian rhythm and reduces muscle growth [Aisbett et al., 2017; Kelu et al., 2019]. Circadian rhythm changes during pregnancy or at a young age are not only able to affect muscle growth, but also have lifelong consequences. The changes in the strategy of energy supply to working muscles, the level of their antioxidant protection, and a decrease in physical endurance occur during the aging process, and the rates of these changes are not the same under different lighting conditions [Vinogradova et al., 2007]. VA has a complex impact on postnatal skeletal muscle function, where it is both an antioxidant and a cell metabolism regulator [Ruiz et al., 2021]. In our study, no differences were found in retinol levels in skeletal muscles between the control and experimental rats, however, some features of age-related dynamics of this vitamin were revealed, which may be associated with metabolic changes in response long-term absence of light and darkness alternation.

The disturbance of the light rhythm has most clearly affected young animals, since photoperiodic stress caused by constant darkness has a negative effect on the rate of physical development [Bishnupuri, Haldar, 2000; Lee, 2007; Yuksel, 2008; Bazhenova et al., 2019]. In our experiment, the absence of daily day/night alternation during embryonic development (in the DD/DD group) raised offspring mortality at birth and in the first month of life, and retarded puberty [Khizhkin et al., 2014]. It is known that constant darkness contributes to depression of gonad maturation, while normal VA content in the diet ensures better development of the reproductive system in rats [Hanai, Esashi, 2011]. We can assume that the effect of constant darkness on VA content in young rats is primarily associated with the functional activity of the pineal gland and melatonin synthesis, which plays a significant role in puberty regulation, the reproductive cycle, and many other physiological processes.

Aging is associated with a disruption of circadian rhythms, which causes changes in photosensitivity in rodents. Studies show that in aging mice, significant changes are observed in SCN

both in light-dark conditions and under constant darkness. At the same time, prolonged exposure to constant darkness masks the effect of aging on the cellular clock of SCN in mice and increases the vulnerability of its circadian ensemble [Nakamura et al., 2015]. It was found that animals living in long photoperiod conditions are physiologically pinealectomized. Decreased melatonin levels lead to metabolic dysfunction and body weight gain [Bishnupuri, Haldar, 2000; Ransom et al., 2014; Ashton et al., 2018; Chitimus et al., 2020]. In addition, melatonin is the only antioxidant whose synthesis declines with age in all species, in contrast to the age-related dynamics of other antioxidants. Thus, the retinol level increases with age, which is associated with an increase in its absorption capacity in aging individuals [Hollander, Dadufalza, 1990; Reiter, 2000]. In our study, no significant differences in body weight were found in rats exposed to different lighting conditions. The retinol content in liver, heart and skeletal muscle increased in old rats in all groups. No effect of photoperiod on the VA level was revealed in 24-month-old rats. The reactivity of the body undergoes a change with age, so resistance to some environmental factors may increase, while to other factors – it may decrease. It is obvious that an age-related decrease in melatonin production against the background of a functional weakening of the pineal gland in combination with prolonged light deprivation leads to a change in central regulation and may have an affect on metabolic processes in tissues.

Conclusions

Our results show that long-term exposure to constant darkness caused the most pronounced changes in retinol content in tissues in early stages of postnatal ontogenesis, which is probably due to a disruption of circadian rhythms, disturbance of metabolic processes, and melatonin level increase. In adult rats, the effect of constant darkness was found in the liver, which may be related to the regulatory role of this organ in VA metabolism. No significant differences in retinol content were found in tissues of aging and old rats kept under different lighting conditions. These data show that the circadian system becomes less reliable during aging. Since retinol acts as one of the mechanisms for the synchronization of physiological processes with the light rhythm, various models of daily cycle disturbance can help to clarify the role of VA in peripheral tissue circadian regulation in mammals. These results can be useful for assessing the physiological state of people living or working in reduced light environments.

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