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CIRCADIAN DISRUPTION AS A FACTOR OF IMPAIRED ADAPTIVE T-CELL IMMUNITY RESPONSE*

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Circadian rhythms are deeply rooted in the biology of virtually all organisms. The pervasive use of artificial lighting in a modern society disrupts circadian rhythms and can be detrimental to our health. About 75% of the world population is exposed to light during the night [1]. Importantly, shift work is a complex lifestyle that to some degree includes not only circadian rhythm and sleep disruption but also altered phase-angle of entrainment and psychosocial stress.

The wide use of artificial lightning among modern society, particularly during night shifts, increases the prevalence of sleep disorders [2], hormone imbalance [3], obesity and cardiovascular diseases [4] in the following population. The mechanisms for these correlations between shift-work exposure and disease are not certainly known; as well as numerous epidemiological studies suggested a correlation between artificial light exposure and health can not determine whether this relationship is causal.

Another group of disorders related to disruption of circadian rhythms shares the common problem of immune response alteration. In mammals, the central circadian pacemaker is located in the suprachiasmatic nucleus (SCN), which entrains peripheral clocks operating at the single-cell level. At the molecular level, these clocks are based on clock genes, which participate in auto-regulatory feedback loops. In the core loop, the transcription factors CLOCK and BMAL1 activate the expression of *Per* and *Cry* genes, whose protein products negatively feedback on their own expression [5]. As *Per* and *Cry* proteins are gradually degraded, the repression on BMAL1 and CLOCK is relieved and the cycle begins afresh with another 24-hour cycle.

Hence, immune cells have the molecular clock machinery and display circadian gene ex-

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pression [6]. The rhythmic functions, such as cytokine production, seem to be under the control of the immune clocks, whereas the number of circulating T-cells appears to be controlled by the central clock via humoral and neuronal signaling [7]. Because the expression of up to 10 % of genes has been suggested to be under the control of the circadian clock, it should not come as a surprise that disruptions in the circadian clock system lead to the onset of various diseases. It is hypothesized that immune clocks are able to gate the integration of central clock's signals to limit or reinforce their effects at certain times of day [8]. Given that, the molecular clock may fundamentally regulate many aspects of our immune system, an understanding of how the clock and immune function intersect may reveal much-needed therapeutic opportunities for chronotherapy of a number of immune related diseases, including cancer therapy regimens [9]. Considering this, investigation of adaptive immunity responses altered by circadian rhythm disruption still retains a high interest [10].

The aim of study is to define effects of circadian rhythm disruption on the adaptive cell immunity, especially by exposition of 24-hour continuous lightning (CL). We have also determined the relationship between the light-induced upregulation of $CD3^+$ co-receptor and proliferation/differentiation processes in $CD4^+/CD8^+$ populations. To provide more complex view, the level of CD95 expression on the surface of $CD3^+$ cells was established, in order to evaluate the effectiveness of death-inducing signaling complex (DISC) formation.

MATERIAL AND METHODS

The experimental study was conducted on mature Chinchilla rabbits which are widely used in immunology research due to short vital cycles and immune plasticity. Animals were exposed to CL, natural day and artificial night lighting (40 w, 2700 K, 390 lm). We organized 5 groups according to the time of withdrawal from the experiment, thus 1st group — 2 month of CL; 2nd group — 4 month of CL, 3rd group — 6 month of CL, 4th group — 12 month of CL. The 5th group is a reference one, containing corresponding age rabbits with unaffected circadian day/night cycle. Due to high sociality of this species and with the aim to reduce general stress level and prevent males from fighting, animals were kept in the spacious pens. Euthanasia performed in appliance with Guidelines for the Housing of Rabbits in Scientific Institutions and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Appendix A, Reference ETS No.123 [11], by using barbiturate overdose. Death confirmed by persistent cessation of heartbeat and respiration, absence of reflexes.

All thymuses were extracted and paraffin-embedded 3–5 μm tissue slides were prepared using the standard protocol. Immunohistochemical identification of T-lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, $CD95^+$) with FITC visualization was carried out on thymus tissue. Prepared slides were examined using Zeiss Axioscope 40 FL, HBO 50. The density of the cells in the different structural-functional zones of the thymus (N/1 mm²) was calculated in 10 fields of view per slide, 5 slides per each CD per each animal group. The images were processed using Zeiss ZEN lite imaging software [12].

Statistical analysis of the following findings was performed using SPSS Statistics; the mean and standard deviation of the normal reference intervals were counted. Data reliability was checked using Student's test; p values less than 0.05 were considered statistically significant.

RESULTS AND THEIR DISCUSSION

The overall counts of $CD3^+$, $CD4^+$, $CD8^+$ positive T-lymphocytes were in a wide variety range (Fig. 1). The 1th group demonstrated slightly lowered cell densities of $CD3^+$ — 0.55 ± 0.14 cells/1 mm²; increase $CD4^+$ density — 1.44 ± 0.15 cells/1 mm² and significant decrease $CD8^+$ density — 0.25 ± 0.04 cells/1 mm² ($p < 0.001$), $CD4^+/CD8^+$ ratio was 3 times increased (Fig. 2).

Previous studies showed changes of immune system organs under unfavorable condition [7, 13] with thymus weight gain due

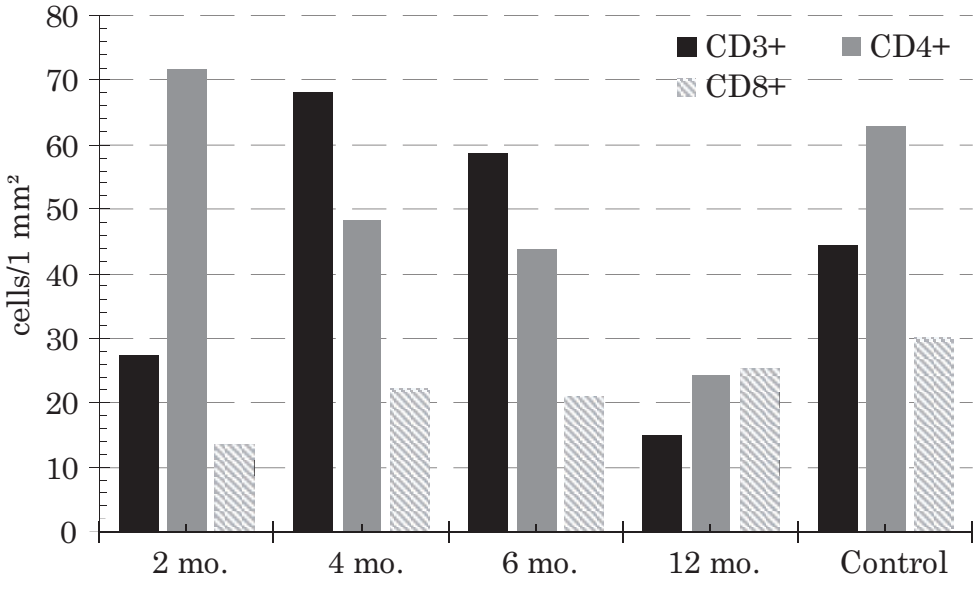

Fig. 1. Cell density in experimental and control groups.

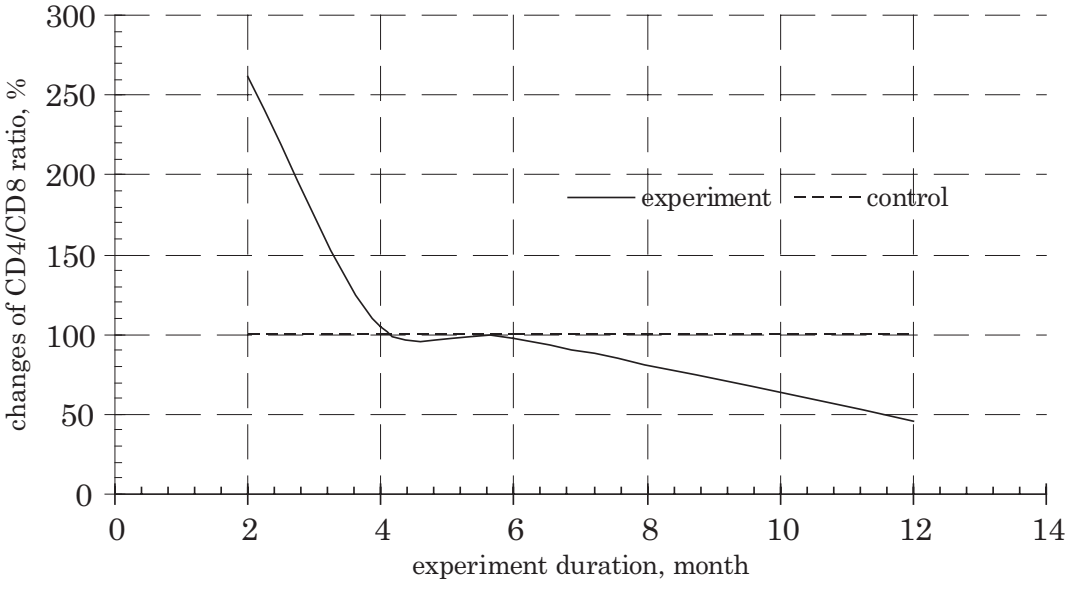

Fig. 2. Dynamic of CD4/CD8 ratio changes.

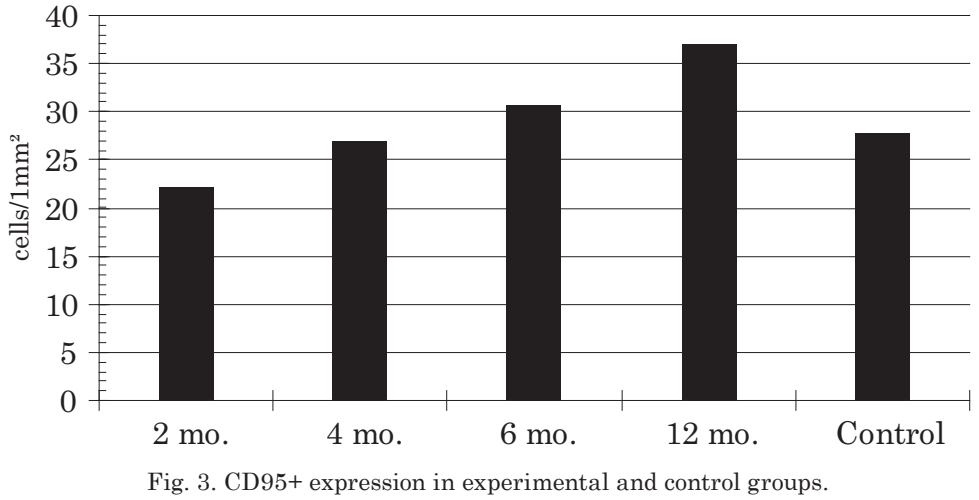

Fig. 3. CD95+ expression in experimental and control groups.

to cortex hyperplasia and an increase of cell density after 2 month of CL. Haertmann F.J. et al. (2016) revealed that narcolepsy, which is considered now as autoimmune disease due to dischronosis, characterize by an increased level of proinflammatory cytokine and T-cells involved in B-cell differentiation. They showed that evaluated expression of T-cell activity from narcolepsy patient was more pronounced in young persons and decreased with the duration of the disease [14].

In the 2nd and 3rd groups cell counts were statistically decreased: $CD4^+$ — 0.97 ± 0.06 cells/1 mm²; $CD8^+$ — 0.45 ± 0.09 cells/1 mm²; and $CD4^+$ — 0.88 ± 0.05 cells/1 mm²; $CD8^+$ — 0.42 ± 0.02 cells/1 mm² respectively, on the contrary we found out that absolute counts of $CD3^+$ positive cells (1.38 ± 0.17 and 1.24 ± 0.08 cells/1 mm²) were significantly increased in comparison with the corresponding reference group ($CD3^+$ — 0.88 ± 0.09 cells/1 mm²). $CD4^+/CD8^+$ ratio was significantly increased ($p < 0.01$) in 2nd group and close to normal in 3rd group.

According to previous studies carried out in V. Danilevsky Institute for Endocrine Pathology Problems of the NAMS of Ukraine, negative influence of continuous lightening leads to hypopinealism and formation of aging pathology [15]. Furthermore, a recent paper described that pinealectomy causes extensive immunosuppression mediated with decrease level of T- and B-lymphocytes and cytokine production. Bedrosian T.A. et al. (2011) suggested that chronic dim light (4 weeks of 5 Lux LAN) is sufficient to alter T-cell-mediated immune response in Siberian hamsters as result changes in melatonin concentration [16]. A negative influence of constant 24h illumination on immune function in crickets, especially leukocyte concentration and lytic activity, is supported by other recent findings [2, 4]. Domingues-Gerpe L. and Rey-Mendes M. showed that chronic immobilization stress considerably increases only the proportion of CD3 immune cells, which have thymus origin. They also observed a decrease in CD4 and CD8 population in the thymus due to long stress influence [17].

The present study confirms recent papers [18] reporting that desynchronization of rhythmic immune parameters and cell proliferation support is induced by circadian dysregulation. Whereas true central SCN and cellular CLOCK/BMAL timekeepers impairment can be seen in the 2nd and 3rd groups, where considerable prevalence of CD3 T-cell co-receptor expression shows discoordination of proliferation and differentiation processes in CD4/CD8 T-cell populations.

The lowest indices of $CD3^+$, $CD4^+$ and $CD8^+$ expression were observed in the 4st group: $CD3^+$ — 0.3 ± 0.04 cells/1 mm²; $CD4^+$ — 0.49 ± 0.05 cells/1 mm². $CD8^+$ cells number (0.51 ± 0.06 cells/1 mm²) was comparable to the control group (CD8 — 0.68 ± 0.05 cells/1 mm²), however $CD4^+/CD8^+$ ratio index was slightly decreased. A significant decrease of $CD3^+$, $CD4^+$ and $CD8^+$ levels in this group shows pronounced immunosuppression and can be a result of premature aging of immune system. Aw D. et al (2008) showed a decrease in cellularity and a slight, but not significant, increase in the number of apoptotic cells in the thymus of older mice [19].

Levels of $CD95^+$ positive cells in our study were slightly increase in the 3rd groups (Fig. 3) and were significantly increased (p0.05) in the 4th group. It's known that stress and aging can induce apoptosis in thymus immature $CD4^+CD8^+$ double positive thymocytes, which are more sensitive to glucocorticosteroid-induced apoptosis [20]. We could support suggesting that T-cell apoptosis is increased with age and activated by the $CD95^+$ signaling mechanism.

CONCLUSION

The presence of artificial light in urban areas is becoming a growing ecological problem. The night light level as low as 5 Lux can lead to changes in melatonin concentration and disturbances in immune system [14]. Our study provides convincing evidence that harmful effects of continuous light exposure cause morphological changes in the thymus. The mechanism of this influence is still unknown, and could be the result of oxidative stress or disturbances in cell proliferation and differentiation, and requires further investigation.

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The study was carried out with the aim to evaluate the effects of continuous lightning (CL) and subsequent circadian rhythm disruption on $CD4^+/CD8^+$ populations of adaptive cell immunity. For further understanding of underlying processes that affect $CD4^+/CD8^+$ proliferation and differentiation, the expression levels of $CD3^+$ and $CD95^+$ cell receptors were evaluated. Chinchilla rabbits were exposed to shift work simulation — 24 hours of CL. We organized 5 time-related groups: 2, 4, 6, 12 months of CL respectively. The 5th group — corresponding control. Thymus derived slides were examined using immunofluorescence assay. A decrease in the expression of $CD3^+$ and $CD8^+$ and an increase in the expression of $CD4^+$ lymphocytes, which was accompanied by an increase in the ratio of $CD4^+/CD8^+$ were found after 2 months of the experiment. After 4 and 6 months of CL, there was an increase in $CD3^+$ expression and a decrease in $CD4^+$ and $CD8^+$ expression. The lowest levels of $CD3^+$, $CD4^+$ and $CD8^+$ were recorded after 12 months of the experiment, which is a manifestation of significant immunosuppression due to premature aging of the thymus. The level of $CD95^+$ lymphocytes was higher in the group after 12 months of CL, indicating the activation of apoptosis in thymocytes caused by circadian rhythms disruption under the influence of chronic light stress.

Keywords: circadian disruption, continuous lightning, T-cell immunity, thymus.

ЦИРКАДНІ ПОРУШЕННЯ ЯК ФАКТОР ПРИГНІЧЕННЯ АДАПТИВНОГО Т-КЛІТИННОГО ІМУНІТЕТУ

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Метою цього дослідження було оцінити вплив тривалого цілодобового освітлення (ЦО), що викликає порушення циркадних ритмів, на популяції $CD4^+/CD8^+$ клітин набутого клітинного імунітету. Для більш глибокого розуміння процесів, що впливають на проліферацію та диференціювання $CD4^+/CD8^+$ лімфоцитів, додатково було вивчено рівень експресії $CD3^+$ та $CD95^+$ рецепторів. Протягом експерименту кролі породи «Шиншила» були піддані впливу 24 годинного освітлення. Організовано 5 груп, в залежності від тривалості експерименту: 2, 4, 6, 12 місяців ЦО відповідно. 5-та група складалася з контрольних тварин. Мікропрепарати тимусу тварин були досліджені з використанням імунофлюоресцентних методів. Після 2-х місяців експерименту відзначали зниження експресії $CD3^+$ та $CD8^+$ та посилення експресії $CD4^+$ лимфоцитів, що супроводжувалося зростанням співвідношення $CD4^+/CD8^+$. Після 4-х та 6-ти місяців ЦО спостерігали посилення експресії $CD3^+$ та зниження експресії $CD4^+$ й $CD8^+$. Найнижчі показники рівня $CD3^+$, $CD4^+$ й $CD8^+$ були зафіксовані після 12-ти місяців експерименту, що є проявом значної імуносупресії, обумовленої передчасним старінням тимусу. Рівень $CD95^+$ лімфоцитів був вищим у групі після 12-ти місяців ЦО, що свідчить про активацію апоптозу у клітинах тимусу, спричинену порушенням циркадних ритмів під впливом хронічного світлового стресу.

Ключові слова: циркадні порушення, тривале освітлення, Т-клітинний імунітет, тимус.