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CIRCADIAN RHYTHMS OF CYTOTOXIC ACTIVITY IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF PATIENTS WITH MALIGNANT MELANOMA

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Aim: **To study circadian rhythms (CR) of cytotoxic activity in peripheral blood mononuclear cells of patients with malignant melanoma were compared with those in healthy men.** ***Methods:*** **The NK-cell and phagocyte cytotoxic activity in five patients with malignant melanoma stage I or II and 12 healthy donors has been assessed by radioimmune assay and NBT-test.** ***Results:*** **The circadian rhythmicity in NK-cells and phagocyte activity in all cancer patients under study has been disrupted. The extent of such disruption tended to increase in patients with more advanced cancer. The most typical alterations were discoordination between the cytotoxicity rhythms of NK-cells and phagocytes (synchronized in healthy persons) and alterations in basic rhythm parameters: phase shifts and amplitude damping.** ***Conclusion:*** **In melanoma patients the significant alteration of CR in NK-cells and phagocytes cytotoxic activity was revealed. In spite of individual variations, the degree of the rhythm disruption basically depended on a disease stage. The alteration of CR phase and amplitude and discoordination between the rhythms of NK- cells and phagocyte were registered in all cases studied.**
Key Words: **malignant melanoma, circadian rhythm, NK-cell activity, phagocytes cytotoxic activity.**

It is known, that most behavioral and physiological functions, including immune resistance, are expressed rhythmically across days and nights. These daily rhythms referred to as circadian provide a temporal frame necessary for adequate homeostasis. CR enable organisms to adapt to daily environmental changes such as light, temperature etc. and serve to synchronize multiple molecular and biochemical processes with each other [1–3]. A number of pathologies are accompanied by alteration in CR. At the same time, CR discordance can lead to such pathological shifts in organism as sleep disorders, cardiovascular diseases, depression and so on. Recent studies show the connection between altered CR and cancer [4–6]. It is established, that alteration of neuro-hormonal circadian coordination accelerates the growth of malignant tumors in mice and serves a poor prognostic factor in cancer patients [7–13], though clinical relevance of hormonal CR for patients outcome may differ according to the hormonal dependency of the tumor [14].

Immune system is involved in control of body homeostasis and in the functional interaction with nervous and endocrine systems [15–17], but chronoimmunological data in oncology are sparse. It is known, that some tumors are associated with alterations in the host CR of various lymphocyte subsets proportions and probably with the altered circadian dynamics of immune functions [8, 9, 18].

Taking into account, that the light is the master environmental pacemaker, controlling circadian dynamics of all physiological parameters, study of immune parameters CR in patients with malignant melanoma is of the special interest, as the light radiation and genetically determined alteration in skin melanocytes photosensitivity are the main ethiologic factors of this cancer. In addition, human malignant melanoma cells express a high-affinity receptors for melatonin — the main chronoimmunomodulator [19, 20].

The data of literature concerning CR of leucocytes cytotoxic activity in patients with malignant melanoma are lacking. Obviously, obtaining such data would be of significant interest. At the same time, carrying out the chronobiological investigations in oncology is highly problematic. It requires the choice of special methodic strategy combining , on the one hand, maximal sparing regimen of cell collecting (which should be as a rule done repeatedly in the day- and night-time) and, on the other hand, still sufficient informativeness of a chosen method as to immune cells activity oscillations during 24 h.

Thus, our work consisted of two phases: at first, an optimal methodical approach was elaborated in studies with healthy donors, and only then the melanoma patients were enlisted to the investigation.

MATERIALS AND METHODS

Blood sampling and cell isolation. CR in five untreated malignant melanoma patients with histologically proven diagnosis and 12 healthy volunteers were investigated according to the following protocol. To study the rhythmicity of cell cytotoxic activity, the blood samples were taken at 9 a.m., 13 p.m., 17 p.m., 21 p.m., 1 a.m. and 5 a.m., effector cells were isolated immediately and NK-cell lytic activity and phagosyte cytotoxic activity were examined. This methodical approach hereafter is referred to as an "*in vivo* method".To study the ability of cells to preserve circadian rhythmicity in vitro, a portion of blood was taken once at 9 a.m. and kept in darkness at 20 °C. The samples for leucocytes isolation and rhythm testing were taken from this portion at the same six time points (the methodical modification was designated an "*in vitro* method").

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Abbreviations used: PBMC – peripheral blood mononuclear cells; NK-cell – natural killer cells; CR – circadian rhythms; NBT – nitroblue-tetrasolium.

To avoid the circannual alteration of circadian rhythms, all presented experiments were carried out in the same season (in summer).

Mononuclear cells were isolated from heparinized blood by centrifugation in ficoll-verografin gradient (ρ = 1,077).

The investigations were approved by Institute Ethic Committee.

NK-cell cytoxicity assay. NK-cell cytotoxic activity of PBMC was tested in a 4 h ^{51}Cr-release assay [21, 22]. The assay was performed using RPMI-1640 medium with 10% heat-inactivated FCS, 2 mM L-glutamine, 50U/ml penicillin and 50μg/ml streptomycin (Sigma, Germany). As a target cells erythromyeloid cell line K-562, labeled with ^{51}Cr, were used. A mixture of effector and target cells (25 : 1) was incubated in U-bottomed 96-well plates (Lenmedpolimer, Russia) in CO_2 incubator at 37 °C. The isotope maximal release and spontaneous release were determined by treatment of the target cells with detergents or medium respectively. All determinations were made in quadruplicates. Radioactivity was counted in a gamma counter (Compugamma, Sweden), and percentage of target-cell lysis was determined according to the formula:

$$\text{Target cell lysis (\%)} = \frac{M_e - M_s}{M_{max} - M_s} \times 100,$$

where: M_e – mean cpm experimental release; M_s – mean cpm spontaneous release; M_{max} – mean cpm maximal release.

Determination of fagocyte cytotoxic activity. Total leucocyte phagocytic actyvity was assessed either with isolated leucocytes [23] or with a whole blood [24] by method of nitroblue-tetrasolium (NBT) reduction. Pirrogenal solution was used to stimulate the cell oxidative burst. The optical density of diformazane formed was measured by spectrophotometer (Specoll, Germany) and microplatephotometer (Labotec, Latvia) (λ = 630 nm). NBT reduction index (k) was determined according to the formula:

$$k\ (\%) = \frac{OD_{sc} - OD_{cc}}{OD_{cc}} \times 100,$$

where: OD_{sc} — optical density of stimulated cells; OD_{cc} — optical density of control cells.

Statistical analyses. Statistical analysis was performed using cosinor method, special mathematic method, developed for biological rhythms analysis [25], linear correlation coefficient and variation statistic methods [26].

RESULTS AND DISCUSSION

The first phase of the work consisted in comparison of cell cytotoxicity assayed by two methods ("*in vitro*" and "*in vivo*") simultaneously. These experiments were carried out only with healthy volunteers. Their aim was to ascertain the possibility to substitute, in studies with melanoma patients, one blood collection procedure for six such procedures (necessary for analysis of a daily rhythm).

The substitution would be acceptable if it turned out that leucocytes retained the rhythmic character of their cytotoxicity in a portion of blood kept for 24 h under *in vitro* conditions. The maintenance of the same rhythmicity in cytotoxicity in a blood sample kept for 24 h *in vitro* was regarded as criterion of acceptance for substitution stated above (as it was shown earlier for CR of T-lymphocytes rosette-forming activity [27, 28]). Then, one portion of blood collected, for example, at 9 a.m. could serve as a source of cells for testing their activity at all other time points.

PBMC isolated from healthy donors showed significant two-phase CR of NK-cell lytic activity both under conditions "*in vivo*" and "*in vitro*" (Fig. 1). Patterns of the cytolytic activity rhythms obtained by these two methods were quite similar. It is still necessary to point out, that the target-cells lysis indices under "*in vitro* method" were at the every time point 5–7% lower than corresponding indices under "*in vivo* method". In both variants of experiments, the highest day time level of NK-cell cytolitic activity was at 13 p.m. and the highest night-time level — at 1 a.m. (In Fig.1–4 the main circadian characteristics — acrophase, mesor, amplitude, were calculated by single cosinor method (data not shown)). The average daily index (mesor) was 34.6% in "*in vivo*" tests and 28.3 % in tests "*in vitro*". Circadian fluctuations in the amplitude of cytolitic activity were insignificantly lower "*in vitro*" than "*in vivo*".

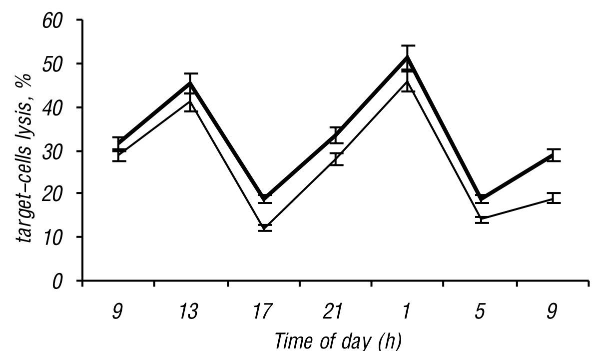

Fig. 1. CR of healthy volunteers' (n = 12) NK-cell activity tested by methods *"in vivo"* (thick line) and *"in vitro"* (thin line)

Comparison of circadian variations in total leucocyte phagocytic activity "*in vitro*" and "*in vivo*" also demonstrated their similarity (Fig. 2). As with the activity of NK-cells, phagocytes showed two-phase circadian rhythms of their cytotoxicity, both "*in vitro*" and "*in vivo*". Again, the daytime highest level of the activity was about 13 p.m. and the night-time highest level — about 1 a.m. Mesor values were 124% "*in vivo*" and 113% "*in vitro*". In average, cytotoxic activity coefficients of mononuclear phagocytes isolated from freshly taken blood samples were 5% higher than in cells obtained from single deposited portion of blood. Finally, CR amplitude of the phagocyte cytotoxic activity did not differ in tests "*in vitro*" and "*in vivo*".

Thus, it may be concluded that the "*in vitro* method" of determination of total leucocyte phagocytic and NK-cells cytotoxic activity of PBMC reproduces rather closely main characteristics of the rhythms, obtained in healthy donors under conditions "*in vivo*". Evaluation of the statistic connection of parameters found "*in vitro*" and "*in vivo*" using linear correlation coefficient (r) confirmed this conclusion: for NK-cell cytotoxicity $r = 0.38$ ($P < 0.01$) and for total leucocyte cytotoxicity $r = 0.34$ ($P < 0.01$).

The aim of next experiments was to find out whether the micro modified methods will be sensitive enough

to register small fluctuations in the cytotoxic activity level caused by CR.

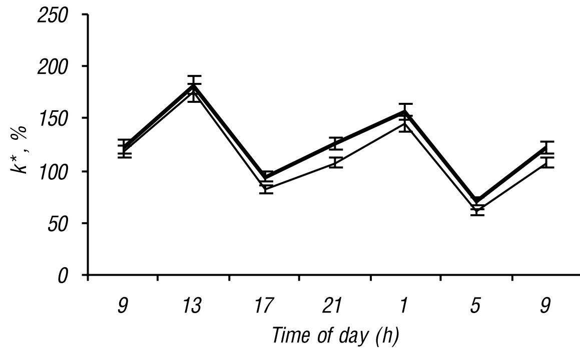

Fig. 2. Circadian rhythms of healthy volunteers' (n = 12) phagocyte cytotoxicity tested *"in vivo"* (thick line) and *"in vitro"* (thin line). k* — NBT reduction index

Circadian patterns of NK-cell cytolytic activity obtained with the isolated PMBC and whole blood (see Fig. 3) were of the same type although the values for a whole blood were on the average 1.4 times lower, than in tests with isolated mononuclear cells. But the statistical analysis (linear correlation coefficient $r = 0.57$, $P < 0.01$) shows a high degree of comparability between obtained results.

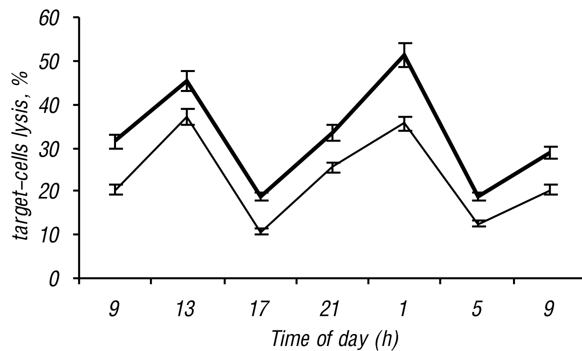

Fig. 3. CR of healthy volunteers' (n = 7) NK-cell activity obtained with isolated PBMC (macro-method, thin line) and a whole blood (micro-method, thick line)

Similarly, comparison of daily rhythms of phagocyte activity in a whole blood and isolated leucocytes revealed their practical identity ($r = 0.69$, $p < 0.05$) (see Fig. 4), though, again absolute values of stimulation indices for isolated leucocytes were on the average 1.5 times higher than in tests with a whole blood.

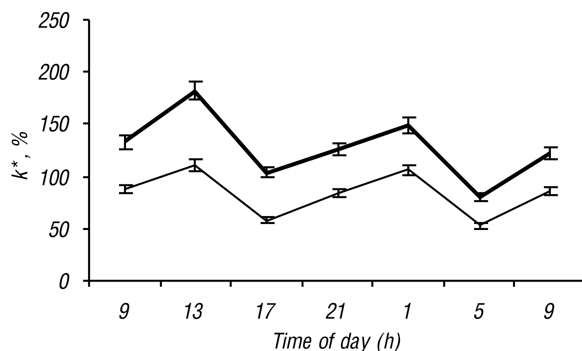

Fig. 4. CR of healthy vounteers' (n = 7) cytotoxic activity in isolated leucocytes (macro-method, thin line) and whole blood (micro-method, thick line k* — NBT reduction index.

Summerazing, the results of this preparative stage of work enabled us subsequently to study CR of malignant melanoma patients by methods "*in vitro*" in their micro modifications, its with the use of a patient's whole blood. And what is more, preservation by leucocytes the CR of their cytotoxic activity *in vitro,* at least for 24 h, confirms presence in human lymphoid cells of self-sustained circadian clock, as we have suggested earlier [27].

Circadian rhythms of NK-cell cytotoxic activity in melanoma patients. It was established that daily rhythms of NK-cell cytotoxicity in patients with malignant melanoma differed significantly from the rhythm of healthy volunteers (Fig. 5). The most typical distinctive features of circadian profiles in the patients were the shifts in a position of time points of NK-cell activity maxima and minima and amplitude changes. A degree of the alterations depended on the disease stage. In one patient with the stage T1, residual features of the two-phase donor-type rhythm were still preserved (Fig. 5.1, Table 1, 1). In two other patients with this stage of the tumor growth (Fig. 5.2. and 5.3., Table 1.2 and 1.3), twofold reduction of the rhythm amplitude in comparison with healthy persons and partial displacement of minimum and maximum time points were observed. Daily average values in patients with disease stage T1 were 1.3 time lower than in healthy donors.

From the example of two patients with stage T2 it is apparent that, depending on the disease progress, more significant modifications in circadian profiles of NK-cell cytotoxic activity occurred with simultaneous amplitude recovering (Fig. 5.4. and 5.5, Table 1.4 and 1.5). In one of the patients, a full inversion of NK-cells activity circadian curve was observed in comparison with the normal rhythm (Fig. 5.4, Table 1.4). It is interesting that similar inversion in daily rhythms of NK-cells and total leucocytes cytotoxic activity of healthy donors we found earlier when comparing the rhythms during two different seasons: in summer time and winter time [29].

In other patient with melanoma T2 (Fig. 5.5., Table 1.5), the loss of two-phase rhythm periodicity was evident in consequence of which the rhythm assumed one-phase character. Both patients of this group had amplitude of NK-cell activity daily oscillations higher than in patients with stage T1 approximating to the donor values. Moreover, the average daily values of NK-cell cytotoxic activity in these patients were 1.5–2.0 times higher than in healthy donors. It seems that rhythm structure simplification in the patients was compensated by amplitude augmentation.

Thus, significant alterations in CR of NK-cell activity occurred in all of the patients with malignant melanoma studied. The similar modifications in CR of immune cell functions were described by Akbulut H. et al. [30] in patients with breast cancer and by Raida M. et al. [31] in patients with advanced gastrointestinal carcinomas. In patients with breast cancer, the alterations in CR of immune cells correlated with those in daily dynamics of melatonine levels and, partly, with cortisole levels. In patients with gastrointestinal carcinomas, the precise

correlation between the hormonal CR and immune cell activity circadian dynamics was absent.

Table 1. Circadian characteristics of rhythms in Fig. 5 evaluated by single cosinor method

Patient	T category	Time of day	Mesor	Amplitude	Acrophase
1	T1	Day time	17.4	6.6	193° (12 : 52)
		Night	14.8	6.1	3° (11 : 25)
2	T1	Day time	13.6	4.8	171° (11 : 25)
		Night	16.6	6.1	2° (0 : 10)
3	T1	Day time	28.6	10.0	199° (13 : 19)
		Night	28.6	9.2	9° (0 : 38)
4	T2	Day time	34.6	10.2	212° (14 : 09)
		Night	51.8	14.6	26° (1 : 45)
5	T2	Day time	38.2	9.6	171° (11 : 25)
		Night	46.6	10.9	2° (0 : 10)

Circadian rhythms of phagocytes cytotoxic activity in melanoma patients. As indicated above, in healthy donors the CR of total cytotoxic activity of leucocytes completely coinsides with that of NK-cells (see Fig. 3, 4): it has the same two-phase character with peaks at 13 p.m. and 1 a.m. (acrophase at 13 p.m.) and with troughs at 17 p.m. and 5 a.m.

The CR of phagocytes cytotoxic activity in melanoma patients has obvious distinctions. The most typical for patients with T1 stage (Fig. 6.1–6.3., Table 2.1–2.3) was amplitude damping: the amplitude of phagocytes activity circadian oscillations in these patients was, on the average, 30% lower than in healthy donors. However, average daily level of phagocyte cytotoxic activity in patients with this stage was equal to maximum level in healthy donors or even above it. Additionally, phase deviations were evident in the rhythms of these patients. In two of them (Fig. 6.1., 6.3, Table 2.1, 2.3), poorly expressed single-phase rhythm was observed, and one of the patients (Fig. 6.1., Table 2.1) had this only preserved peak near 17 p.m. (i.e., at the time when PBMC from healthy donors exhibit minimum of cytotoxic activity).

As far as it is possible to judge from only two cases included in this investigation the main charateristic feature of CR in the cytotoxicity of melanoma patients with T2 stage (Fig.6.4, 6.5, Table 2.4, 2.5) was amplitude augmentation (comparing to patients with T1 stage) against the background of general rhythm simplification, i.e. the changes were similar to such for NK-cells activity.

Table 2. Circadian characteristics of rhythms in Fig. 6 evaluated by single cosinor method

Patient	T category	Time of day	Mesor	Amplitude	Acrophase
1	T1	Day time	105.2	31.8	199° (13 : 16)
		Night	101.2	28.0	15° (1 : 20)
2	T1	Day time	102.4	35.2	196° (13 : 07)
		Night	106.4	31.2	9° (0 : 37)
3	T1	Day time	132.2	28.7	192° (12 : 51)
		Night	122.0	21.2	13° (0 : 55)
4	T2	Day time	99.4	32.2	204° (13 : 38)
		Night	29.8	10.4	7° (0 : 29)
5	T2	Day time	184.2	70.3	193° (12 : 54)
		Night	125.4	48.8	2° (23 : 49)

Analysing melanoma patients' rhythms of phagocytes and NK cells cytotoxic activity in parallels (see Fig. 5, 6), it is clear that synchronism of the rhythms between these two leucocyte subpopulations which is characteristic for healthy donors (Fig. 1–4) is completely lost in all of the patients.

Thus, in the present study it is shown for the first time that in patients with a primary untreated melanoma deep alterations in circadian rhythms of NK-cell and total leucocyte phagocytic activity occur. The separate preparatory part of the work was designed to choose and approbate in chronobiological experiments more sparing and less damaging methodical approaches that allowed to realize such serial 24 hr-long investigations, at least on the restricted group of cancer patients.

In spite of small number of the melanoma patients examined, the general conclusion about dramatic changes in cytotoxic activity rhythms with alteration of all principal circadian parameters and loss of synchronism between rhythms of NK-cells and phagocytes is deemed rather founded because the changes were apparent in all five patients. Unlike that, observations as to peculiarities of the rhythm deterioration depending on disease stage 1 or 2 are purely tentative being based on a comparison of groups with only three and two patients, correspondingly.

Mechanisms of alteration in CR of lymphoid cell functions are difficult to explain. There are attempts to link such alterations with an endocrine system dysfunction. Data of human and animal studies suggest that in a healthy organism both immune cell subpopulations number and activity exhibit CR that seem to correlate with daily fluctuations of the cortisol level [15, 16]. But in cancer patients the alteration of this cortisol rhythmicity is observed only when tumors are hormone dependent [14, 31]. It is also known that secretion of melatonin is disturbed in patients with some tumors [33, 34]. Based on these results, some authors even recommend the melatonine application in cancer therapy [35, 36].

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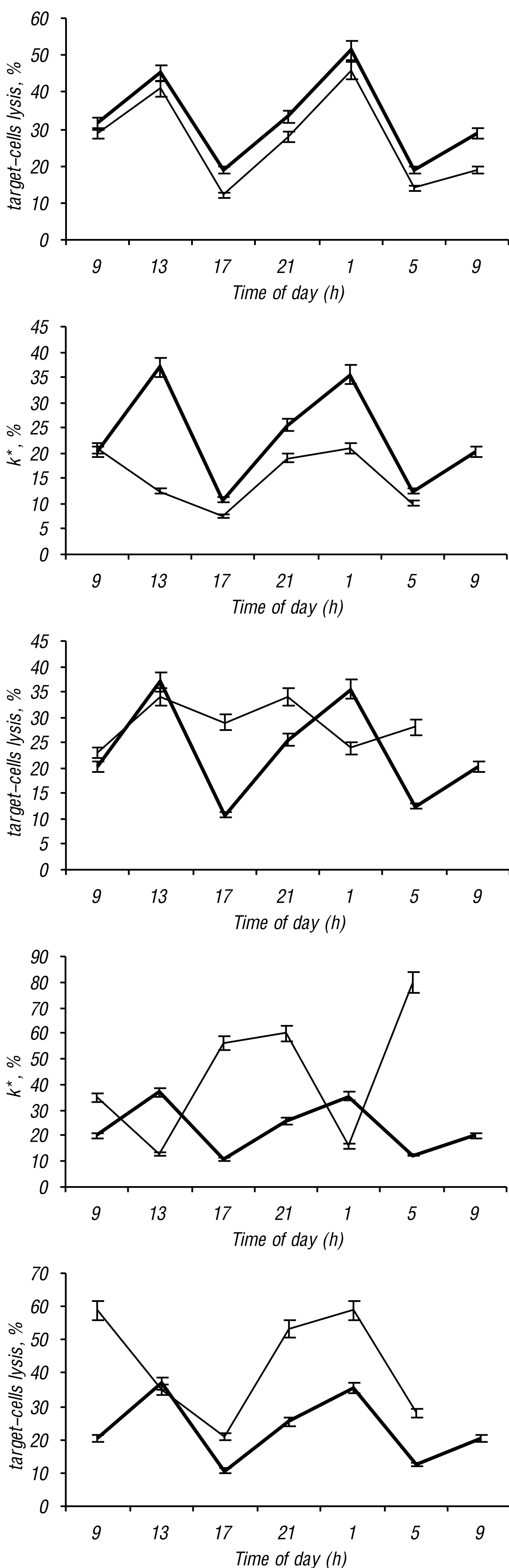

Fig. 5. CR of NK-cell cytotoxic activity in 5 patients with malignant melanoma (thick line).The typical healthy donors' rhythm (thin line, n = 7) is shown for comparison

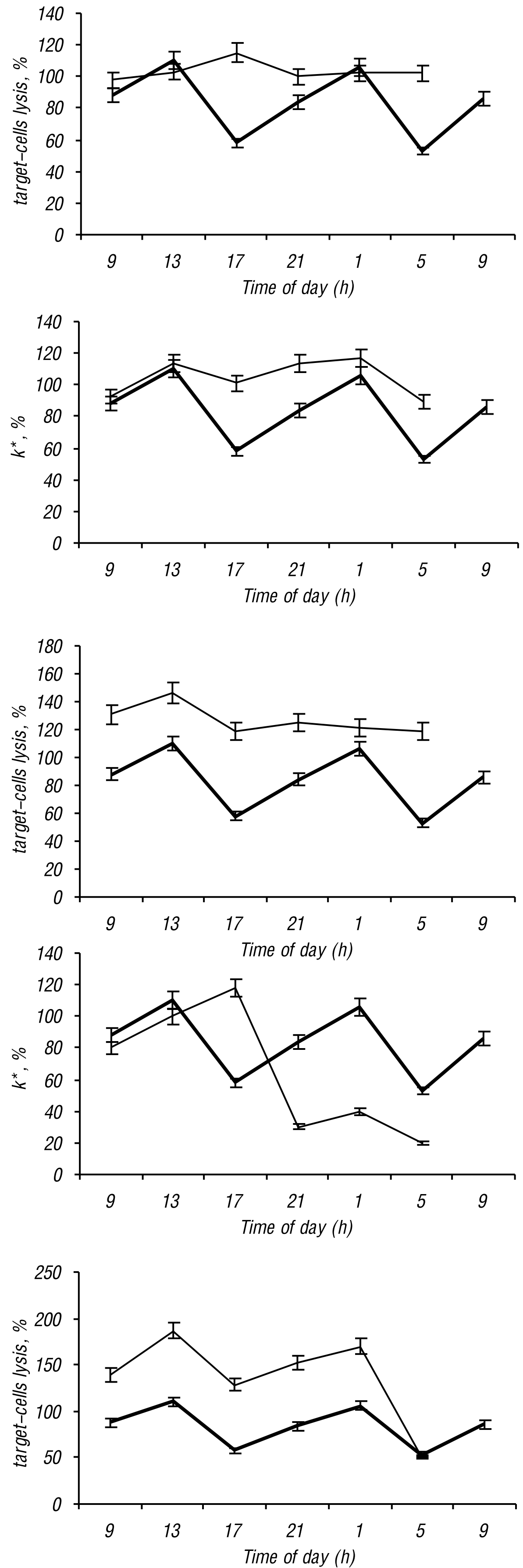

Fig. 6. CR of phagocyte cytotoxic activity in 5 patients with malignant melanoma (thick line). The typical healthy donors' rhythm (thin line, n = 7) is shown for comparison
***k** — koefficient of NBT-reduction

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ЦИРКАДИАННЫЕ РИТМЫ ЦИТОТОКСИЧЕСКОЙ АКТИВНОСТИ МОНОНУКЛЕАРНЫХ КЛЕТОК ПЕРИФЕРИЧЕСКОЙ КРОВИ БОЛЬНЫХ ЗЛОКАЧЕСТВЕННОЙ МЕЛАНОМОЙ

Цель: **изучить циркадианные ритмы (ЦР) цитотоксической активности мононуклеарных клеток периферической крови (МКПК) больных злокачественной меланомой по сравнению с таковыми здоровых доноров.** ***Методы:*** **активность НК-клеток и МКПК, выделенных из крови 5 больных со злокачественной меланомой I и II стадий и 12 здоровых доноров, оценивали при помощи радиоиммунного метода и NBT-теста.** ***Результаты:*** **установлено, что у онкологических больных нарушена нормальная циркадианная ритмичность активности НК-клеток и МКПК, причем степень выраженности таких нарушений возрастает параллельно с прогрессией заболевания. К наиболее типичным изменениям относятся дискоординация между цитотоксическими ритмами НК-клеток и мононуклеарными фагоцитами (синхронизированными у здоровых доноров) и изменения основных параметров ритма: сдвиг фаз и смещения амплитуды.** ***Выводы:*** **у больных меланомой выявлены значительные изменения ЦР НК-клеток и МКПК, причем степень нарушения ритма зависит от стадии заболевания. Во всех изученных случаях выявлены изменения фаз и амплитуды ЦР, декоординация ритмов НК-клеток и МКПК.**
Ключевые слова: **злокачественная меланома, циркадианный ритм, активность НК-клеток, цитотоксическая активность мононуклеарных клеток.**

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