ХРОНОБИОЛОГИЧЕСКИЙ ПОДХОД К ИЗУЧЕНИЮ ФИЗИОЛОГИЧЕСКОЙ АКТИВНОСТИ *CANDIDA* SPECIES

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Резюме. Изучение биологических свойств в течение суток позволяет выявить ритмометрические маркеры, которые можно использовать для дифференциальной диагностики возбудителей при разных состояниях пациента. Данные закономерности изучены на примере клинических изолятов *C. albicans*, *C. tropicalis* и *C. krusei*, выделенных из вагинальной микробиоты при кандидозном дисбиозе. Контролем служили эталонные штаммы из американской коллекции типовых культур (АТСС). Физиологические свойства детально изучены на примере биопленкообразования дрожжевых патогенов. Биологическую активность пленкообразования *Candida* sp. смотрели в течение двух суток с 4-часовым интервалом, в зимнее время года. Для экспериментов использовали суточные культуры, что соответствовало максимальной адгезии их на поверхности стекла. Хронометраж исследований подразумевал получение по оцениваемой функции 6 измерений в сутки с 3-5-кратным повторением условий эксперимента. Для выявления цикличности изучаемого параметра, данные статистически обработаны по Стьюденту, непараметрическими методами с применением критерия Манна–Уитни и методу наименьших квадрантов.

В ходе экспериментов выявлено наличие пленкообразующей активности грибов в течение суток ($p < 0,05$) и обнаружить общие закономерности проявления свойств у представителей всех изучаемых видов. Доказано, что основными ритмометрическими параметрами имеющие диагностическое значение являются период ритма и амплитудно-фазовая стабильность. Установлено, что суточная динамика биопленкообразования C. albicans 24433 ATCC характеризовалась ультрадианным (около 12-часовым) вкладом ритма в утреннее – 04:00 и вечернее время – 16:00. У *C. non-albicans* ATCC выявлены достоверные циркадианные (околосуточные) ритмы активности адгезии к поверхности стекла. У клинических изолятов дрожжей, выделенных из женского репродуктивного тракта при кандидозной патологии, динамика биопленкообразования характеризовалась достоверными ультрадианными (около 12-часовыми) гармониками, что имеет важное биологическое значение, определяющее устойчивость к внешним воздействиям и способность к адаптивному ответу на периодические раздражители.

Использование хронобиологического метода, на наш взгляд, открывает новые перспективы при изучении физиологии *Candida* sp., так как дает возможность прогнозировать динамику состояния

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Образец цитирования:

М.В. Николенко, Н.В. Барышникова, О.И. Малишевская, Е.М. Васева «Хронобиологический подход к изучению физиологической активности Candida species» // Медицинская иммунология, 2023. Т. 25, № 3. С. 563-568.
doi: 10.15789/1563-0625-CAT-2750

For citation:

M.V. Nikolenko, N.V. Baryshnikova, O.I. Malishevskaya, E.M. Vaseva "Chronobiological approach to study the physiological activity of Candida species", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 563-568.
doi: 10.15789/1563-0625-CAT-2750

DOI: 10.15789/1563-0625-CAT-2750

микроорганизма и учитывать особенности срочной и долговременной адаптации к разным факторам внешней среды. Выявление суточных ритмов биопленкообразующей активности у различных штаммов *Candida* sp., открывает возможность управлять жизнеспособностью бактериально-грибковых ассоциаций и прогнозировать их устойчивость к различным антимикробным средствам.

Ключевые слова: хроном, биоритмы, грибы рода Candida, биопленки, физиологическая активность, амплитудно-фазовая стабильность

CHRONOBIOLOGICAL APPROACH TO STUDY THE PHYSIOLOGICAL ACTIVITY OF *CANDIDA* SPECIES

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Abstract. Rythmometric markers can be identified within a day during the study of biological characteristics in order to be used for differential diagnostics of pathogens of different patients' physical condition. These principles are based on analysis of clinical isolates *C. albicans*, *C. tropicalis* and *C. krusei* allocated from the vaginal microbiota at *Candida* dysbiosis condition. Control examples were the master samples from the American Type Culture Collection (ATCC). Detailed research was conducted on physiological characteristics through the formation of biofilms by yeast pathogens. Biological activity of *Candida* sp. biofilming was observed within 2 days with 4 hours interval in winter. Daily cultures were used for the experiment to correspond to their maximum adhesion to the glass surface. It was important to obtain 6 measurements per day with 3-5 times repetition of experiment conditions during the specified timeline. In order to determine the periodicity of the parameters studied, the data was statistically processed by Student's t-test, using Mann–Whitney criteria and nonparametric method of least square method.

It was found out that biofilming activity during 24 hours ($p < 0.05$) of fungi exists and that all species have many principles in common. It was attested that the main rhythmometric parameters of diagnostic significance are the rhythm period and amplitude-phase stability. It was found that the daily dynamics of C. albicans 24433 biofilm formation from American Type Culture Collection was characterized by an ultradian (about 12-hours) contribution of the rhythm in the morning – 4 A.M and in the evening – 4 P.M. Significant circadian (approx. daily) rhythms of adhesion glass surface activity were revealed in *C. non-albicans* from American Type Culture Collection. The dynamic of biofilm formation isolates of yeast from female reproductive organs with *Candida* pathology was characterized by reliable ultradian (about 12-hour) harmonics which biological significance defines resistance to external impact and the ability to adaptively respond to periodic stimuli.

To sum up, implementation of the chronobiological approach has opened up new prospects for studying the physiology of *Candida* sp., as it enables us to predict the dynamics of microbial states and takes into account the specificity of emergency and long-term adaptation to different environmental factors. The detection of the circadian rhythm of biofilm formation activity of different *Candida* sp. strains provides the possibility to manage the vitality of the Society of Bacteria and Fungi and predicts its resistance to various antibiotics.

Keywords: chronome, biorhythms, Fungi of the genus Candida, biofilms, physiological activity, amplitude-phase stability

Introduction

Biofilm formation is one of the main survival strategies of bacteria. Bacterial-fungal microsymbiocenosis acquires protection from various physical, chemical, and biological antimicrobial factors. Therefore, we can consider a biofilm as one of the forms of persistence of microorganisms [3]. Up to 80% of infectious diseases [15] and polyantibiotic resistance of microorganisms [5] are associated with biofilm formation. A number of papers review the importance of biofilms for various fields of scientific research and applications, showing an increased interest in this problem [11], as well as information on a large number of methods for studying, culturing, and indicating biofilms in vivo and in vitro [9].

In recent years, dynamic methods for studying the formation of biofilms have been widely used, namely, the Robinson apparatus in its various modifications, laboratory fermenters, and the flow method [6]. The maximum approximation to the conditions of living

systems is the main advantage of dynamic methods of biofilm formation [13]. Fluorescence, confocal scanning laser and electron microscopy are innovative technologies that revealed the heterogeneous structure of bacterial-fungal biofilms. The genes encoding adhesion proteins of *Candida* sp. [12] were studied and the phases of biofilm formation were described: adhesion, formation of the extracellular matrix, and formation of a mature biofilm consisting of single cells and mycelium [4]. In their works, Al-Fattani M. et al. [1] studied the chemical composition of the *C. albicans* and *Candida* tropicalis (*C. tropicalis*) biofilm matrix. The main structural components were proteins, carbohydrates, hexosamine, and phosphorus.

In the last decade, biofilms in bacterial-fungal populations have been studied by the method of fluorescent hybridization to determine the location of mRNA in the cells that form biofilms. It was used to identify persister cells responsible for the survival of the population under the influence of factors of various nature [2].

The methods used in scientific research to study biofilm formation are complex and require specialized equipment, a vivarium, which creates difficulties in real clinical practice in identifying biofilms and results in a false impression of the low frequency of their formation.

Another group of methods is based on the creation of static conditions for the cultivation of microorganisms, which are convenient, highly productive and visual. The authors proposed a modification of the method for dynamic studying biofilm formation on plastic dishes during the day [14]. From this point of view, the chronobiological approach acts both as a methodological principle and as a technique [7].

Aim: studying the physiological activity of *Candida* species using chronobiological approaches.

Materials and methods

Seventeen isolates of C. albicans, *C. tropicalis* and *C. krusei* isolated from the reproductive tract of women diagnosed with candidal dysbiosis were the object of the study. Repository reference strains of *Candida* sp. obtained from the American Type Culture Collection (ATCC) C. albicans 24433, *C. tropicalis* 750, and *C. krusei* 6258 were used as a control. These types of fungi were chosen as a model since in the vast majority of cases (85-90%) the causative agent of candidiasis is C. albicans, the most pathogenic and significant species in clinical practice. Among other species of *Candida* sp., predominantly *C. tropicalis* with 3-5% and *C. krusei* with 1-3% are of clinical significance. Recently, the number of diseases caused by *C. non-albicans* fungi has increased [1].

The fungi were identified by a set of features: cultural properties, sensitivity to antifungal drugs by the disk diffusion method, chlamydospore formation, morphogenesis ability, type of filamentation, and saccharolytic and biochemical activities [8].

The paper presents a modified method for assessing biofilm formation proposed by O'Toole G.A. et al. [12]. At the first stage of the study, 24-hour cultures were obtained by growing them on a Sabouraud nutrient medium with tellurite at a temperature of 37 °C, which corresponded to the beginning of the stationary phase of fungal development. To obtain a biofilm, glass test tubes P-2-14-120 washed with a chromium mixture were used. The initial 0.5 McFarland concentration of microorganisms was prepared using a densitometer. To obtain a working concentration of 1.5×10^3 CFU/mL, the cultures were titrated with physiological saline, after which they were added to test tubes with Sabouraud Dextrose Broth and incubated in a thermostat at a temperature of 37 °C. After incubation, to remove the planktonic phase of the culture, the contents of the tubes were taken with a pipette without touching the walls of the vessel and gently washed twice with distilled water. To quantify the biofilm volume, 2 mL of gentian violet was added. After 45 minutes, the dye was carefully poured off and the walls of the test tube were repeatedly washed with distilled water. To extract the dye, 2 mL of 96% ethanol was added to each test tube for 15 minutes.

The kinetics of biofilm formation was studied by changes in the light transmittance and optical density on a KFK-3-01 spectrophotometer at a wavelength of 540 nm; measurements were taken after 24, 36, 48, 72 and 96 hours. 96% ethanol was used as a control. Gentian violet extracted with ethanol from the biofilm was placed into disposable plastic macro cuvettes $10 \times 10 \times 45$ mm in size, 4 mL in volume (optical path length was 10 mm). The temperature of the measured liquids corresponded to room temperature. To obtain statistically reliable data, the experiment was repeated five times. Experimental data were statistically processed in Primer of Biostatics Version 4.03 by Stanton A. Glantz 1998, Microsoft Office Excel 2010, with a given certainty $p < 0.05$.

In the second series of experiments, the authors studied the biofilm formation activity of *Candida* sp. within two days with a 4-hour interval, the fourth lunar phase, in the winter season. A 48-hour culture of fungi was used, which corresponded to their maximum adhesion on the glass surface. Eleven experiments were performed; 486 measurements were obtained.

The data were processed by the least squares method with a given significance of certainty $p < 0.05$ [10]. The main parameters of the rhythms were determined: the rhythm contribution ($T = 24$; $T = 12$), mesor, amplitude and acrophase of the rhythm.

The Mann–Whitney test compared the differences between unrelated samples (experiment – control, collection and hospital cultures). The analyzed differences were considered significant at $p < 0.05$.

Results and discussion

All *Candida* sp. isolates had a characteristic growth rate. The adhesive properties of fungi of the *Candida* genus depended on the phase of their development. The glass surface of the test tube in the nutrient medium was covered with primary biofilm after 12 hours of culturing. The first (logarithmic) section (12-24 hours) of the kinetic curve corresponded to the stage of reversible and irreversible microbial adhesion, the duration of which did not depend on the nature of the microorganism. It was experimentally found that 48 hours was the most optimal time for studying the biofilm formation: the light transmittance was the highest, since the fungal cell walls inside the biofilm sorbed the dye, gentian violet, to the maximum extent. Therefore, the ability of micromycete cells to form conglomerates is significantly higher in the stationary growth phase than in the logarithmic one ($p < 0.05$). The cultivation period of 72-96 hours was characterized by a decrease in the activity of biofilm formation by fungi. Apparently, their cells lost their mobility and began to intensively secrete extracellular polymers, forming a polymer matrix [11]. Lisovskaya S.A., 2008, obtained similar results when observing 1-4 day cultures using the technique of growing biofilms on a nitrocellulose surface [8].

The chronobiological method used by the authors in the second series of experiments revealed the presence of biofilm-forming activity of fungi during the day ($p < 0.05$) and found common patterns in the manifestation of properties in representatives of all studied species.

The daily dynamics of *C. albicans* biofilm formation was characterized by an ultradian (about 12-hour) rhythm with an acrophase in the morning and evening. Significant circadian rhythms of adhesive activity were found for *C. non-albicans*. The maximum values of the indicator for *C. tropicalis* were recorded in the evening, and for *C. krusei* at night. The presence of circadian rhythms in the spectral composition of microorganisms simultaneously with ultradian rhythms indicates an increase in the adaptive capabilities of microorganisms. Average daily indicators (mesor) of biofilm formation did not exceed 1.5±0.05, which made it possible for us to infer that the activity of all the studied cultures was low (Table 1).

For all clinical isolates of *Candida* sp. isolated from the reproductive tract of women diagnosed with candidal dysbiosis, the dynamics of biofilm formation was characterized by significant ultradian variations, with 8 and 12-hour fluctuations. The maximum values were recorded in the early morning from 03.09 to 04.18 and in the evening from 18.45 to 20.20 (Table 2). On the contrary, ultradian rhythms characterize the variability of periods, which determines the ability

TABLE 1. RHYTHMOMETRIC PARAMETERS OF BIOFILM FORMATION OF COLLECTION STRAINS OF C. SPECIES

Cultures	Period (day)	Rhythm contribution, %	Mesor M±m	Amplitude	Acrophase, hour
		24-hour 12-hour		24-hour 12-hour	24-hour 12-hour
C. albicams 24433	1	34.3 58.6*	1.50±0.04	0.30±0.04 0.50±0.01	04.18 16.40
	2	38.0 60.8*	1.40±0.07	0.20±0.09 0.40±0.05	05.35 17.03
C. tropicalis 750	1	69.0* 13.4	1.40±0.05	0.40±0.05 0.10±0.02	17.10 06.00
	2	86.4* 9.3	1.50±0.04	0.20±0.01 0.10±0.01	16.02 04.04
C. krusei 6258	1	78.5* 2.6	1.30±0.05	0.30±0.04 0.10±0.01	20.00 04.03
	2	54.7* 34.1	1.30±0.06	0.40±0.03 0.30±0.04	20.00 03.18

Note. *, $p < 0.05$.

TABLE 2. RHYTHMOMETRIC PARAMETERS OF BIOFILM FORMATION OF C. SPECIES ISOLATES FROM BIOLOGICAL MATERIAL OF WOMEN WITH CANDIDAL DYSBIOSIS

Culture	Period (day)	Rhythm contribution, %	Mesor M±m	Amplitude	Acrophase, hour
		24-hour 12-hour		24-hour 12-hour	24-hour 12-hour
C. albicans	1	11.5 81.8*	2.60±0.01*	0.50±0.02 2.30±0.19*	18.45 03.12
	2	8.3 90.4*	2.40±0.01*	0.20±0.01 1.90±0.39*	20.00 07.54
C. tropicalis	1	17.8 78.3*	1.7±0.3	0.7±0.2 1.5±0.3	04.00 20.25
	2	25.3 65.2*	1.7±0.2	0.3±0.1 1.0±0.3*	03.34 19.20
C. krusei	1	30.2 53.7*	1.7±0.1	0.5±0.1 1.5±0.1*	04.18 20.12
	2	26.8 54.8*	1.6±0.4	0.9±0.2 1.3±0.4	04.06 19.40

Note. *, $p < 0.05$.

to adaptively respond to periodic stimuli and resist external influences [7].

It has been experimentally established that the average daily biofilm formation rates for C. albicans are significantly higher than for *C. non-albicans* ($p < 0.05$). The revealed regularity is consistent with the data obtained in experiments on silicone models. Confocal scanning laser and electron microscopy shows that C. albicans forms a quantitatively larger and structurally more complex biofilm than *Candida* non-albicans [14]. The extracellular matrix of *C. albicans* consisted of 57.0% glucose, while the biofilm of *C. tropicalis* was dominated by hexosamine. Biofilm formations of C. albicans were more easily separated from plastic surfaces by treating them with the beta-1,3-glucanase enzyme than that of *C. tropicalis* [8].

Analysis of the chronoinfrastructure of the studied function of clinical isolates and reference strains of *Candida* sp. showed a change in the contribution of rhythm, mesor and amplitude. The activity of biofilm formation increased in the direction "reference strains – clinical isolates". The Mann–Whitney test was 29 for C. albicans, 26 for *C. tropicalis*, and 30 for *C. krusei* ($p < 0.05$). The chronobiological approach expands the understanding of yeast physiology. In the course of the study, we found that the contribution of rhythm and the amplitude-phase characteristic of biofilm formation are rhythmometric markers of strain pathogenicity. These criteria are stationary and distinguish pathological disorders from adaptive changes [7].

Conclusion

The proposed macrometric method solves the problem of accelerating and simplifying the quantitative assessment of the process of biofilm formation and increases sensitivity as it eliminates errors associated with the use of polystyrene material. The ease of its implementation makes it accessible to any laboratory.

In our opinion, the chronobiological method opens up new perspectives in the study of the physiology of *Candida* sp., as we can predict the dynamics of the biological activity of a microorganism and take into account the features of immediate and long-term adaptations to various environmental factors. The detection of daily rhythms of biofilm formation activity in various strains of *Candida* sp. opens up the possibility of controlling the viability of bacterial-fungal associations and predicting their resistance to various antimicrobial agents.

Acknowledgments

The research was funded by a grant from Tyumen State Medical University in the field of biomedical and biopharmaceutical technologies No. 8210053 dated August 12, 2021. The team of authors would like to thank Tyumen State Medical University for their support in conducting the research.

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Поступила 13.04.2023
Отправлена на доработку 14.04.2023
Принята к печати 17.04.2023

Received 13.04.2023
Revision received 14.04.2023
Accepted 17.04.2023