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DETERMINATION OF CHANGES IN THE PATHOGENICITY FACTORS ACTIVITY OF THE ORAL CAVITY MICROFLORA IN PATIENTS WITH SECONDARY ADENTIA DEPENDING ON THE TAME OF DAY

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ABSTRACT

Investigation of biological properties in microorganisms remain relevant to the current stage of dentistry development. The aim of this work was to study the adhesive and enzymatic activity of oral microbioma representatives with determination of ability to form biofilms at different times of day Materials and methods. The total of 35 patients with partial secondary adentia were involved in the study. Studies in 35 patients who were in the stages of examination and preparation for the dental implantation surgery on partial secondary adentia (included small single or bilateral defects). Ability to form biofilms and the enzymatic activity was studied in Staphylococcus aureus, Streptococcus mutans, Candida Albicans and Hafnia Alvei at 9, 12, 15 and 18 hours. Results. It has been established that the high activity of enzymes and the ability to form biofilm occurs during times when medical manipulations are the most frequently carried out - at 1200 and 1500. The peaks of reduced pathogenicity factors production were recorded at 9.00 and about 18.00. Conclusions. The obtained results can be used for optimal planning of rehabilitation manipulations (dental implantation operations) in the oral cavity in individuals with secondary adentia.

INTRODUCTION

The existence of a person at each level is tightly connected with microorganisms. The microbiom of the mouth cavity is a unique and important component of a human microbiom, which has a direct impact on human health from metabolism to immunity and from birth to a long-term exposure throughout life [1, 2]. Today it is known that one of the main strategies of the bacteria survival, both in the environment and macroorganisms are the formation of microbial communities - biofilms.

The mouth cavity is not an exception either, where conditions for biofilms creation are ideal, namely: a wet medium, a constant temperature with a stable source of nutrients [3, 4]. In the composition of bacteria, bacteria cells are combined with complex intercellular bonds that regulate the expression of genes in different parts of biofilms and at various stages of their development, resulting in a population of biofilm bacteria, they are considered as a functional analogue of a multicellular organism [5, 6, 7]. Also, today it is known that microbiom, as well as any organism, depends on biophysical conditions and has a circadian rhythmicity of its functioning and it helps the “host” to function in accordance with the circadian clock [8, 9].

For the current stage of dentistry development, studying the microorganisms' biological properties to deepen the ideas of the mechanisms of inflammatory processes' emergence and development in the oral cavity are relevant, with the identification of the laws of their oscillations during the day.

Information on biorhythms of microorganisms, which are representatives of oral microbiom and dental diseases pathogens, is isolated, controversial, not systematized. Therefore, the aim of this work was to study the adhesive and enzymatic activity of oral microbioma representatives with determination of their ability to form biofilms at different times of day in patients with partial secondary adentia.

MATERIALS AND METHODS

The total of 35 patients aged 35 to 57 years (18 women and 17 men) were involved in the study, they did not have clinical signs of the mucous membrane diseases in the oral cavity and periodontal tissues. The mouth cavity of the specified persons was slammed, the CPU index amounted to a mean of 5.64±0.15 standard units. All the patients did not have bad habits (taking alcohol, smoking), did not consume antibiotics and any other antimicrobial preparations over the past 6 months. All the patients were at the stages of examination and preparation for the dental implantation surgery with partial secondary adentia (included small single or bilateral defects). The cause of teeth loss was a complication of caries.

Collection of material for microbiological examination was performed in the morning under conditions of morning hygiene and eating at least 2 hours before taking meals with sterile applicators in accordance with the rules of asepsis to avoid contamination of the sample with foreign microflora. After collection, the material was immersed in the transport medium and delivered to the laboratory in accordance with current modern requirements. Regarding the extraction and identification of microorganisms, the preparation of microorganisms suspensions with a certain concentration of microbial cells was performed by means of B1Q electronic device Densi-La-Meter (PLIVA-Lachema a.s., Czech Republic) by the McFarland scale according to the instructions for the device.

Enzymatic identification was performed using Microlatest® kits (Erba Mannheim, France), which are designed for standard identification using micromethods and permits identification of most clinically important microorganisms in a relatively short time. Microorganisms such as Staphylococcus aureus (S. aureus), Streptococcus mutans (Str. Mutans), Candida albicans (C. albicans) were selected for the study on the basis of their dominance in the oral cavity. Although Hafnia alvei (H. alvei) is not a constant representative of the oral microbiome, the presence of this microorganism is associated with immune suppression and drug resistance, so we considered it appropriate to study changes in its physiological activity.

The optical density was measured using a microplate reader "Multiskan EX" (type 355), which is a photometer with replaceable filters and is able to perform standard photometric measurements.

Studies of the biofilms formation were performed by determining the ability of bacterial strains to adhere to the surface of polystyrene tablets [10]. Cultures were grown on suspension media recommended for each family of bacteria. The obtained cultures were washed with suspension media,

which are also individual for each family of bacteria. Measurements of the optical density of the initial bacterial suspension were performed with a Densi-La-Meter instrument (Erba Mannheim, France). The number of inoculated planktonic cells was counted with a photometer "Multiskan EX 355" at a wavelength of 540 nm and was expressed in conventional units of optical density.

Вивчення адгезивної активності мікроорганізмів проводили за методикою В.І.Бриліс (1986). Оцінку адгезивних властивостей бактерій здійснювався за формулою:

The study of the microorganisms' adhesive activity was performed according to the method of V.I. Brilis (1986). Assessment of the adhesive properties of bacteria was carried out according to the formula:

$$AIM = \frac{AA \times 100}{CA},$$

where AIM is the adhesion index of microorganisms, AA is the average adhesion (average number of bacteria attached to one erythrocyte), CA is the coefficient of erythrocyte participation in adhesion (percentage of erythrocytes that had adhered bacteria on their surface). Regarding the criteria for assessing the adhesive properties, the microorganism was considered non-adhesive at AIM ≤1.75; low-adhesive – 1.76 to 2.5; medium adhesive – from 2.51 to 4.0; and highly adhesive at AIM more than 4.0.

Study of enzymatic activity of microorganisms.

Determination of DNAse activity was performed by inoculating the bacterial culture on Petri dishes with 1.5% agar, which was prepared in Martin broth with the addition of DNA and calcium chloride solution. After incubation in a thermostat at 37^0C for 18 hours, DNAse activity was assessed by measuring the diameter of the clarification zones around the colonies: the clarification zone 5 - 8 mm corresponded to the high activity of the strain, 3 - 4.9 mm - medium, 1 - 2.9 mm - weak activity, and less than 1 mm - no activity.

Determination of lecithinase activity: daily agar cultures were inoculated on Hottinger's agar containing lecithovitelin and incubated at 37^0C for 24 hours. Assessment of the strain activity was performed depending on the diameter of the iridian crown around the colonies.

Hemolytic activity was determined by inoculating a daily agar culture on 4% Hottinger agar containing a 5% suspension of rabbit erythrocytes. Petri dishes were incubated in a thermostat at 37^0C for 24 hours. The strains were considered hemolytically active in the presence of a zone of hemolysis around the colonies.

Proteolytic activity was determined on milk agar: meat peptone agar with addition of 10% pasteurized milk. The magnitude of proteolytic activity was judged by the size of the lysis zones around the colonies of S. aureus on milk agar.

Reliability of the obtained values was checked using Student's t test. Statisica 64 version 10 was used for statistical processing of results.

RESULTS AND DISCUSSION

In determining the adhesive activity of S.aureus, Str. mutans, H. alvei, C. albicans was found to be time-dependent, with a peak activity of 12^{00} and 15^{00}, and clinical strains of H. alvei and C. albicans at 18^{00} compared with an activity of 9^{00} when all strains had an index of mean adhesion. The most highly adhesive strains were registered at 12^{00}: C. albicans - AIM was 9.7 ± 0.39 and H.alvei - AIM was 8.7 ± 0.14 (table 1). The isolates of

S. aureus and Str. mutans about 9^{00} were of low adhesion. Clinical strains of H. alvei and C. albicans at 9^{00} and S. aureus and Str. mutans at 18^{00} were of mean adhesion.

Table 1. Adhesive properties of clinical strains depending on the time of day

Isolates	Adhesion index	Time of observation			
		9^{00}	12^{00}	15^{00}	18^{00}
Staphylococcus aureus	AA	1.8 ±0.14*	6.4 ±0.12*	4.2 ±0.14*	2.6 ±0.12*
	CA	73.2 ±0.81*	92.6 ±0.52*	82.1 ±0.26*	76.9 ±0.48*
	AIM	2.5 ±0.15*	6.9 ±0.18*	5.1 ±0.13*	3.4 ±0.18*
Streptococcus mutans	AA	1.6 ±0.14*	6.9 ±0.16*	4.6 ±0.18*	2.4 ±0.22*
	CA	74.8 ±0.24*	94.2 ±0.23*	84.6 ±0.42*	78.1 ±0.34*
	AIM	2.1 ±0.14*	7.3 ±0.16*	5.4 ±0.14*	3.1 ±0.21*
Hafnia alvei	AA	2.1 ±0.17*	7.5 ±0.15*	5.9 ±0.18*	4.2 ±0.28
	CA	76.1 ±0.32*	86.1 ±0.34*	82.3 ±0.26*	82.6 ±0.42*
	AIM	2.8 ±0.16*	8.7 ±0.14*	7.2 ±0.18*	5.1 ±0.23*
Candida albicans	AA	2.4 ±0.19*	7.8 ±0.29*	6.4 ±0.28*	4.8 ±0.29*
	CA	72.8 ±0.26*	80.3 ±0.38*	79.4 ±0.36*	84.9 ±0.38*
	IAM	3.3 ±0.26*	9.7 ±0.39*	8.1 ±0.34*	5.7 ±0.31*

Note. * - the marked values of the mean value are significantly different from the corresponding index of the reference strains at $p<0.001$.

The value of the erythrocyte participation coefficient in adhesion varies depending on the ability of the pathogen to adhere and the time of day: the highest adhesion coefficient was found in Str. mutans (94.2 ± 0.23 units) and S. aureus (92.6 ± 0.52 units) at 12^{00}, and the lowest rate was in C. albicans (72.8 ± 0.26 units) and S. aureus (73.2 ± 0.81 units) at 9^{00}.

The mean adhesion was the lowest in all clinical strains at 9^{00} with maximum

values at 12^{00} and a gradual decrease at 15^{00} and 18^{00}.

When determining the DNAse isolates activity of S. aureus revealed high DNAse activity in 12 (80.0%) isolates of 12^{00} and 11 strains (73.3%) of 15^{00}. And at 9^{00} only 2 (13.3%) strains of 15 had high DNAse activity, and 13 (86.7%) were weakly active (table 2).

Table 2. DNAase activity of isolates

Time of day	Strains	DNAase activity					
		High		Moderate		Weak	
		abs	%	abs	%	abs	%
9-00	*S.aureus*	2	13.3	0	0	13	86.7
	Str.mutans	3	20.0	2	13.3	10	66.7
	H.alvei	4	26.7	3	20.0	8	53.3
	C.albicans	4	26.7	4	26.7	7	46.6
12-00	*S.aureus*	12	80.0	3	20.0	0	0
	Str.mutans	14	93.3	1	6.7	0	0
	H.alvei	13	86.7	2	13.3	0	0
	C.albicans	15	100	0	0	0	0
15-00	*S.aureus*	11	73.3	4	26.7	0	0
	Str.mutans	12	80.0	3	20.0	0	0
	H.alvei	12	80.0	1	6.7	2	13.3
	C.albicans	13	86.7	2	13.3	0	0
18-00	*S.aureus*	0	0	0	0	15	100
	Str.mutans	0	0	12	80.0	3	20.0
	H.alvei	0	0	10	66.7	5	33.3
	C.albicans	0	0	12	80.0	3	20.0

It should be noted that at 18^{00} in all strains of S.aureus weak DNAse activity was recorded, in addition, no clinical strain had high activity on this basis.

The maximum values of DNAse activity of Str.mutans isolates were detected at 12^{00} in 14 (93.3%) strains and in 12 strains (80.0%) at 15^{00}. At 9^{00}, only 3 (20.0%) strains out of 15 had high DNAase activity, 2 strains (13.3%) showed mean DNAse activity, and 10 (66.7%) were weakly active. It was found that about 1500 14 strains (93.3%) had DNAse activity, and 3 (20.0%) - moderate. At 18^{00} in 12 (80.0%) strains of Str. mutans recorded mean DNAse activity and 3 strains (20.0%) had weak activity by this trait.

As for the DNAse activity of isolates of H.alvei and C.albicans, it was found that 13 strains (86.7%) of H.alvei and all isolates of C.albicans had a high DNAase activity at 12^{00} and 12 (80.0 %) of H. alvei isolates and 13 (86.7%) clinical strains of C. albicans at 15^{00}. At 18^{00}, the moderate DNAse activity was recorded in most strains: 10 (66.7%) H. alvei isolates and 12 80.0%) strains of C. albicans.

When determining the lecithinase activity of 15 clinical strains of H.alvei had a high degree of 2 strains (13.3%) at 9^{00}, moderately active were also 2 strains (13.3%), were weakly active 8 strains, which is 53.3% (table .3). All clinical strains of S. aureus and C. albicans had the highest degree of lecithinase activity at 12^{00}.

Table 3. Lecithinase activity of clinical strains

Time of day	Strains	Lecithinase activity					
		High		Moderate		Weak	
		абс	%	абс	%	абс	%
9-00	*S.aureus*	0	0	2	13.3	13	86.7
	Str.mutans	0	0	5	33.3	10	66.7
	H.alvei	2	13.3	2	13.3	8	53.3
	C.albicans	1	6.7	3	20.0	11	73.3
12-00	*S.aureus*	15	100	0	0	0	0
	Str.mutans	14	93.3	1	6.7	0	0
	H.alvei	13	86.7	2	13.3	0	0
	C.albicans	15	100	0	0	0	0
15-00	*S.aureus*	11	73.3	4	26.7	0	0
	Str.mutans	10	66.7	3	20.0	2	13.3
	H.alvei	12	80.0	1	6.7	2	13.3
	C.albicans	10	66.7	2	13.3	3	20.0
18-00	*S.aureus*	0	0	2	13.3	13	86.7
	Str.mutans	0	0	12	80.0	3	20.0
	H.alvei	0	0	8	53.3	7	46.7
	C.albicans	0	0	10	66.7	5	33.3

Analyzing the results for the determination of plasma coagulase activity in S. aureus, it was found that all clinical strains had this pathogenicity factor regardless of the time of day. All clinical strains of S. aureus had proteolytic and hemolytic activity at 12^{00} and 15^{00}, and at 9^{00} and 18^{00} the production

of aggression enzymes was recorded in 66.7% of S. aureus cultures.

Studies of the daily dynamics in the formation of dense biofilms by clinical strains of H.alvei, C.albicans, Str.mutans and S.aureus and the production of planktonic cells with the ability to form new biofilms revealed that the maximum rate of formation of daily dense biofilms with the production of planktonic cells of clinical strains was registered at 12^{00} and 15^{00}. The tendency to lower density of biofilms formation with decrease in activity of planktonic cells production was observed at 9^{00} and 18^{00} (fig. 1).

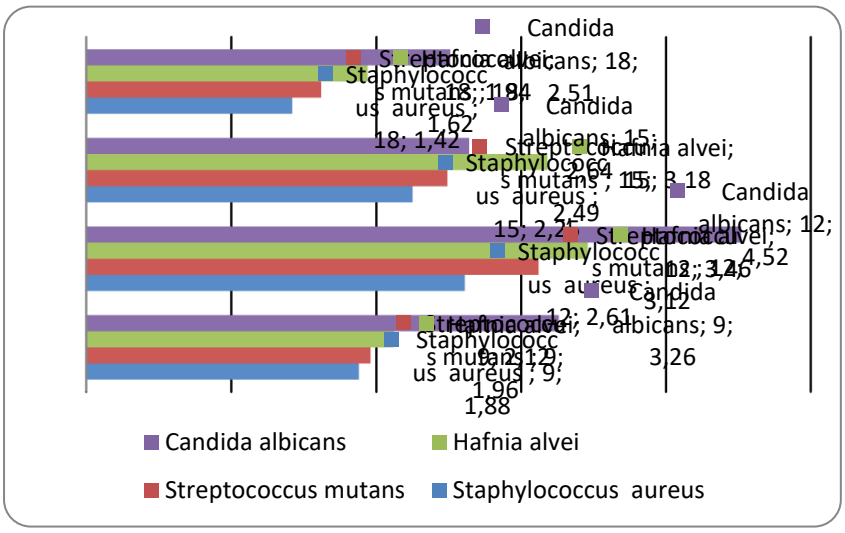

Figure 1. Changes in the optical density of clinical strains biofilms of H.alvei, C.albicans, Str.mutans and S.aureus during the day.

Decreases in the activity of planktonic cell production by isolates were registered at 9^{00} and 18^{00}, compared to the biofilms formation by reference strains, in which fluctuations in the density of biofilms and planktonic cell products did not differ significantly during the day (fig. 2).

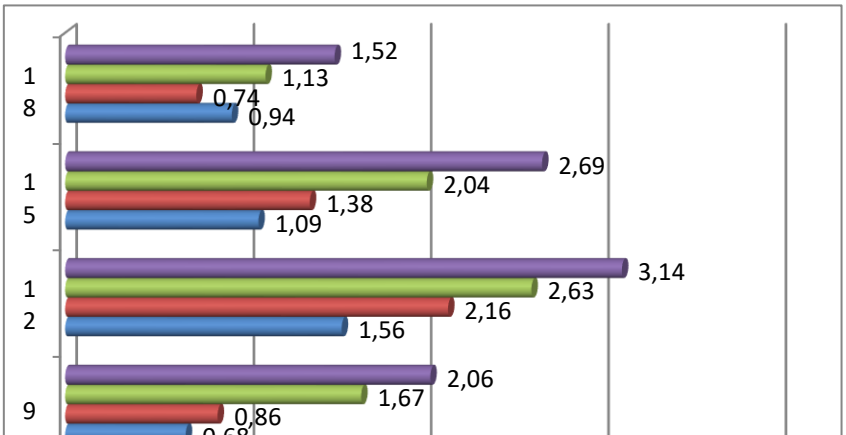

Fig.2. Changes in the optical density of planktonic cells of H.alvei, C.albicans, Str.mutans and S.aureus during the day.

A similar trend is observed in all isolates in the dynamics of planktonic cell production depending on the time of day.

Thus, the assessment of changes in the indices of physiological activity in the oral microflora in patients with secondary adentia permitted to determine the adaptive responses of microbes to the action of the clock factor. As a result of determining the daily dynamics of producing pathogenic enzymes and the ability to biofilm formation in leading clinical strains of microorganisms in persons with secondary adentia, it was found that high enzyme activity occurred at times when medical manipulations were most often performed - at 12^{00} and at 15^{00}, 9^{00} and at 18^{00}.

It is now known that the protection of a microorganism from the action of pathogenic microorganisms is individual and associated, among other reasons, with circadian rhythms or biological clock, which significantly affect both the microflora and the immune system. In particular, in the case of inflammatory diseases, circadian rhythms are likely to play a role in terms of susceptibility, clinical expression and outcome [11]. But if circadian rhythms are the subject of active study in basic research, its relevance in the medical field, particularly in dentistry, remains, unfortunately, limited. [12, 13, 14].

However, in our opinion, the consideration of circadian rhythms can be the basis for optimal planning of rehabilitation manipulations in the oral cavity (e.g., dental implant surgery in persons with secondary adentia) at the time of their implementation and appointment of concomitant drug therapy, as well as to assess the results of interventions, risk of complications. Regarding the latter, taking into account the circadian rhythms of microorganisms can provide not only a general assessment of the risk, but also the probable biological origin of this risk. Thus, the above may open new opportunities for the development of preventive and personalized methods of dental patients treatment on the basis of time.

CONCLUSIONS

1. The maximum rate of daily dense biofilms formation with the production of planktonic cells of clinical strains was registered at 12^{00} and 15^{00}, and lower density of biofilms with reduced activity of planktonic cells was observed at 9^{00}

and 18^{00}, which should be taken into account when planning dental implantation.

2. Formation of bacterial biofilms depends on the adhesive properties of pathogens with active production of planktonic cells, which indicates the adaptive response of microorganisms with the formation of aggression factors and multidrug resistance to antimicrobial drugs selected for the study of clinical strains.

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