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Expression of periferal core molecular clock genes in oral mucosa depends on the chronotype in patients with maxillofacial cellulitis

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ABSTRACT

Introduction: Accurate determination of the patient's chronotype is one of the problems of personalized medicine. Recent studies have shown that determining of the expression of timing genes is a valuable method that can help gain molecular insight into a patient's intrinsic circadian timing. Odontogenic cellulitis is very common pathology. Since acute inflammatory diseases are an urgent pathology, the time of surgical intervention can correspond depend on the time of the patient's hospitalization.

Materials and methods: The level of mRNA expression of peripheral circadian clock genes *clock* and *bmal1, per1, cry1* in buccal epithelial cells in patients with odontogenic purulent inflammatory diseases of maxillofacial area in the morning and evening was investigated.

Results: During analyzing the results of the mRNA expression study of the genes of the negative regulatory link of the peripheral molecular clock, per1 and cry1, in patients with Cellulitis of the maxillofacial area, a significant decrease ($P = 0.0003$) in the mRNA expression level of the cry1 gene by 2.61 times in the evening compared to its morning mRNA expression values.

Conclusion: The obtained data indicate that in patients with odontogenic purulent inflammatory diseases of the maxillofacial area with an evening chronotype, a violation of the expression profile of the *per1* gene in the cells of the buccal epithelium is noted, which is manifested by an increase in its evening expression in comparison with patients with a morning chronotype.

1. Introduction

Research in recent years has shown that circadian clocks develop a central role in maintaining tissue homeostasis, disrupting which can lead to the development or progression of diseases.[1]

The presence of the main components of peripheral clocks was demonstrated in different tissues of the oral cavity, head and neck,[2] but the specific functions of the corresponding peripheral clocks in the tissues of the oral cavity, head and neck and the mechanisms underlying in the implementation of these functions are still widely unknown. At the same time, a number of studies demonstrate that a dysfunctional clock mechanism is involved in the development of oral cavity cancer and juvenile skeletal hypoplasia of the lower jaw.[3] It has been established that the chronotypical characteristics of a person affect the life expectancy[4] and the development of diseases.[5,6]

Accurate determination of the patient's chronotype is one of the problems of personalized medicine. Recent studies have shown that determining of the expression of timing genes is a valuable method that can help gain molecular insight into a patient's intrinsic circadian timing.[7]

It has been demonstrated that gene expression in the tissues of the oral mucosa has essential daily fluctuations and can be considered as a biosample as a circadian biomarker.[8] It was noted that in healthy volunteers with daytime activity, the main peaks of expression of *per1* and *bmal1* time genes in the mucous membrane of the oral cavity coincide with cell cycle markers: p53 (marker of G1 phase) and cyclin B1 (marker of M-phase), respectively. This supports the circadian coordination of cell cycle events in the oral mucosa.[9] The researchers found that the human clock genes *clock, tim, per1, cry1* and *bmal1* are formed in the oral mucosa and skin, with a circadian profile that corresponds to that found in the suprachiasmatic nuclei and peripheral tissues of rodents, and their functional significance is important for humans. *Per1, cry1* and *bmal1*

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are rhythmic, peaking in the early morning, late afternoon, and night, respectively, whereas *clock* and *tim* are not.[10]

Odontogenic purulent inflammatory diseases (cellulitis) of maxillofacial localization are a very common pathology, and make up from 40% to 60% of the total number of stocks hospitalized in maxillofacial hospitals. The issue of optimizing surgical and drug therapy of this pathology is an important medical and social problem, which is determined by the high risk of further complications and the long time required for the treatment and rehabilitation of such diseases.[11] Since odontogenic cellulitis are an urgent pathology, the time of surgical intervention can correspond depend on the time of the patient's hospitalization. Thus, there may be an inconsistency between the patient's chronotype and the time of the surgical intervention, which justifies the need to conduct our study.

Knowledge of the mechanisms of functioning of the circadian clocks of the oral cavity, head and neck will contribute to the development of a therapeutic strategy in maxillofacial surgery, which provides for the possibility of influencing more than one specific target,[3] will allow to more effectively determine the time of surgical interventions and medical manipulations,[12] which can significantly improve treatment tactics and prognosis in patients with maxillofacial cellulitis, as well as to identify new targets for their therapy.

The purpose of this study is to determine the connection between the expressions of the peripheral molecular clock core genes in the buccal epithelium of patients with maxillofacial cellulitis with certain chronotype advantages for the further development of schemes of personalized medical interventions.

2. Materials and methods

The study was conducted on 16 patients (males) with maxillofacial Cellulitis, aged 35–60 years, who were undergoing inpatient treatment in the maxillofacial surgery department of the Poltava Regional Clinical Hospital, Ukraine.

2.1. Chronotype determination

Patients with a definite morning and a definite evening chronotype, were determined according to the Horn-Ostberg questionnaire, and were selected for the group.[13] The Horn-Ostberg test consists of 23 multiple-choice questions related to the interviewee's daily schedule, each having four or five response options. The main purpose of this questionnaire is to measure whether a person's circadian rhythm produces peak activity in the morning, in the evening, or between. The study included only people of the morning chronotype and the evening chronotype.

2.2. Sampling, mRNA extraction and real-time PCR

Expression of circadian clock genes was determined in cytological samples of the buccal epithelium witch were taken at the beginning of the treatment (before the surgical intervention). Sampling was carried out taking into account previous studies that revealed a significant 12/24-h fluctuation in the expression of the circadian clock genes *per1* and *bmal1*[10] at 08-00 in the morning and at 20-00 in the evening.

Sampling of the buccal epithelium was carried out using an Interdental bristles (Dontodent, Germany) with a tapered plastic bristle and a blunt end. For the contact of the bristles with the cheek mucosa, the plastic holder of the cytological brush was slightly bent to ensure sufficient lateral pressure on the mucous membrane. After it, rotational movements of the brush were performed at the place where the biological material was taken for 10 s in one direction with simultaneous pressure on the cheek mucosa. At the same time, significant irritations and bleeding were avoided. After taking the material, the brush was immediately immersed in RNA stabilizing solution (ThermoFisher, USA) at room temperature. Taken and stabilized samples were stored at −20 °C for further research.

Total RNA was isolated from a biological sample using a set of reagents for isolation and purification of RNA (QIAGEN, Germany). A set of reagents were used to obtain the cDNA to carry out reverse transcription reaction (QIAGEN, Germany). The level of mRNA expression of *bmal1, clock, per1* and *cry1* genes in buccal epithelium samples was determined by polymerase chain reaction in the "real-time" mode (Real-time PCR). The real-time PCR was performed on the CFX96TM Real-Time PCR Detection System (Biorad, USA) when using SybrGreen I. Quantitec®sybr-Green I PCR Kit (QIAGen, Germany). Specific oligonucleotide primers are listed in Table 1.

Amplification was conducted after a 30-s denaturation at 95 °C followed by 45 cycles of incubation at 95 °C for 15 s and at 60 °C for 20 s. The *β-actin* gene was used as a reference gene. The relative delta Ct method was used for data analysis.

2.3. Statistical analysis

Differences in gene expression levels across time and chronotype groups, and across time by chronotype interaction were calculated by one-way ANOVA & post-hoc Bonferroni test and two-way ANOVA & post-hoc Bonferroni test, respectively, using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA).

3. Results

3.1. Expression of peripheral circadian clock genes in patients with odontogenic inflammatory diseases of soft tissues of maxillofacial area

The level of mRNA expression of peripheral circadian clock genes *clock* and *bmal1, per1, cry1* in buccal epithelial cells in patients with maxillofacial cellulitis in the morning and evening is shown in Fig. 1.

The difference between the expression of the mRNA of the positive regulatory unit gene - *bmal1* in the evening was statistically significantly higher than the morning expression of mRNA ($p = 0.0075$). During analyzing the results of the mRNA expression study of the genes of the negative regulatory link of the peripheral molecular clock, *per1* and *cry1*, in patients with cellulitis of the maxillofacial area, a significant decrease ($P = 0.0003$) in the mRNA expression level of the *cry1* gene by 2.61 times in the evening compared to its morning mRNA expression values.

The peripheral molecular clock is in close relationship with the central pacemaker, the main activator of which is light, which forms the chronotype preferences of a person. It also has a significant impact on the course of reparative processes in the organism. So, the level of mRNA expression of *clock, bmal1, per1, cry1* genes in patients with odontogenic purulent inflammatory diseases of soft tissues of maxillofacial area with different chronotypes was investigated.

Table 1
Primers to determine gene expression.

Gene	Sequence of primers	Reference
clock,	F: 5′- AAAATACTCTCTACTCATCTGCTGG-3′	[14]
	R: 5′- ATGGCTCCTTTGGGTCTATTG-3′	
bmal1	F: 5′- CTGGCTAGAGTGTATACGTTTGG-3′	
	R: 5′- GGTCACCTCAAAGCGATTTTC-3′	
per1	F: 5′- ATTCCGCCTAACCCCGTATGTGACC-3′	
	R: 5′- GTGTGCCGCGTAGTGAAAATCCTCTTGT -3′	
cry1	F: 5′- TTACACTATGCTCATGGCGAC-3′	
	R: 5′- GTGCTCTGTCTCTGGACTTTAG-3′	
β-actin	F: 5′- TCCACCTTCCAGCAGATGTG -3′	
	R: 5′- GCATTTGCGGTGGACGAT -3′	

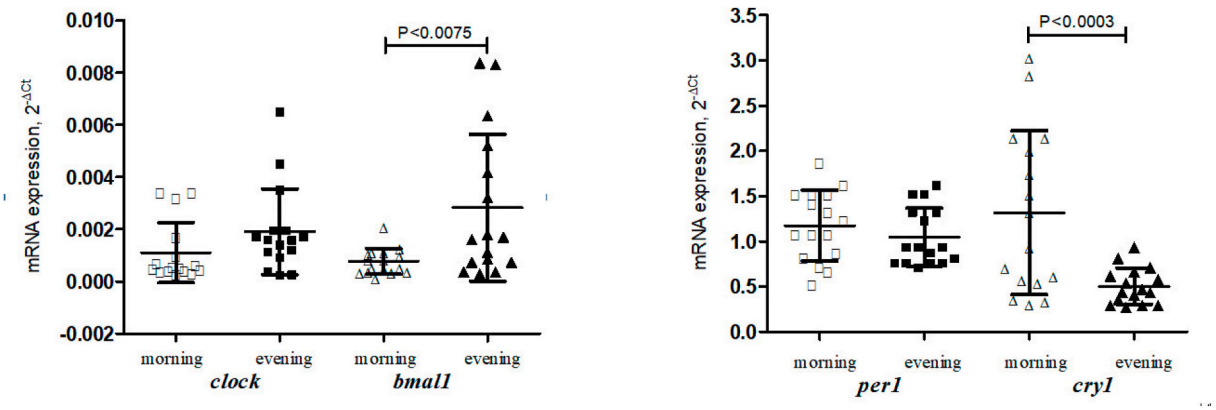

Fig. 1. Comparison of mRNA expression levels of peripheral circadian clock genes *clock, bmal1, per1, cry1* of oral mucosa in patients with maxillofacial cellulitis in the morning and evening.

3.2. The diversity in gene expression of the positive and negative link of regulation in patients with odontogenic purulent inflammatory diseases

The diversity in gene expression of the positive link of regulation of the peripheral molecular clock *clock* in individuals with cellulitis of the maxillofacial area with a morning chronotype in the morning and in the evening had no significant differences. We have established probable differences between the expression of the *bmal1* gene in the morning and in the evening in persons with a morning chronotype in the direction of an increase in its evening indicator (Fig. 2).

During studying the difference between the morning and evening expressions of the genes of the negative regulatory link of the peripheral molecular clock, *per1* and *cry1*, in persons with maxillofacial cellulitis with a morning chronotype, statistically significant decrease in the expression level of the evening index of the *cry1* gene was observed ($P < 0.05$). Also, the expression level of the mRNA *per1* gene in the evening was significantly lower than in the morning ($P < 0.006$). We also have determined and compared mRNA expression levels of *clock, bmal1, per1,*

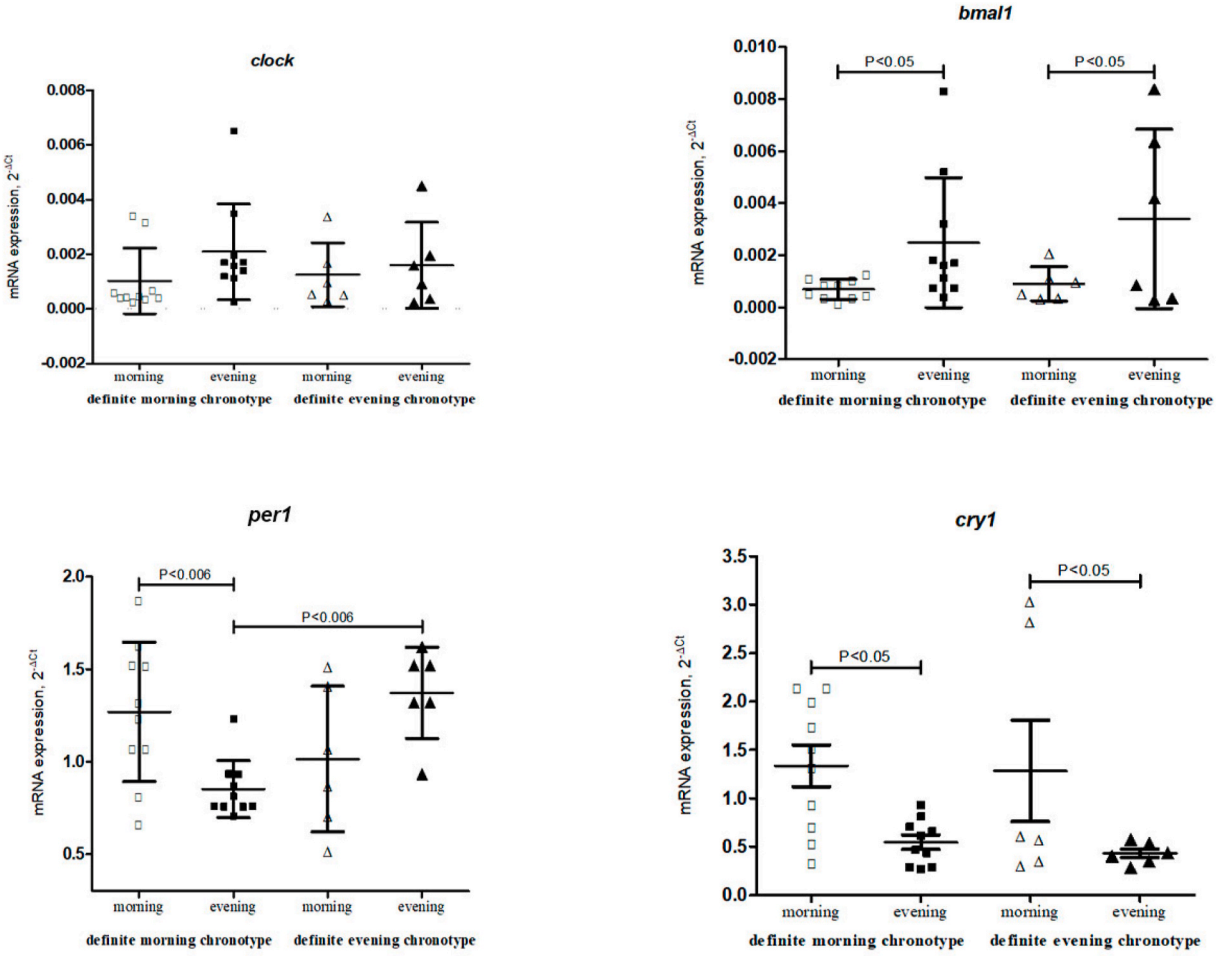

Fig. 2. Comparison of mRNA expression levels of peripheral circadian clock genes *clock, bmal1, per1, cry1* of oral mucosa in patients with maxillofacial cellulitis in the morning and evening depending on the chronotype.

cry1 genes in patients with maxillofacial cellulitis with an evening chronotype in the morning and evening. During comparing morning and evening mRNA expression of *clock, bmal1, per1, cry1* genes in patients with maxillofacial cellulitis with an evening chronotype in the morning and evening, it was noted a statistically significant increase in *bmal*1 gene mRNA expression in the evening compared to its morning expression ($P < 0.05$).

Expression of the *cry1* gene in the morning was significantly increased compared to the level of expression in the evening ($P < 0.05$). The values of the morning expression of *per1* did not differ significantly from the evening expression in the group of patients with the evening chronotype, but there was also a statistically significant increase in its evening expression in the group of patients with the evening chronotype compared to patients with cellulitis of the maxillofacial region who had the morning chronotype ($P < 0.006$). We did not find a statistically significant difference in this group of patients between the morning and evening mRNA expressions of the other genes of the peripheral molecular clock that were studied.

4. Discussion

Our study has several limitations. The small sample size of this study limited the statistical power to find genes with circadian rhythmic expression and to assess correlations between genes expression and the development of odontogenic purulent inflammatory diseases of maxillofacial area. Future studies with a larger sample size are needed to confirm our findings. Additionally, we did not take into account the influence of such factors as lifestyle, diet, intensity of physical activity, and sleep quality. The prognosis for further investigation is to study the correlation between the expression of core clock genes and the adenophlegmones of maxillofacial localization.

The rhythmic expression of clock genes is the result of a balance between the positive and negative branches of the feedback loop, with *bmal1* and *clock* belonging to the positive branch, and *per1, per2, cry1* and *cry2*, to the negative branch.[3] During studying the expression of the genes of the peripheral molecular clock in the cells of the buccal epithelium, we have found that in patients with cellulitis of the maxillofacial area with a morning chronotype, there was noted a significant decrease in the levels of evening mRNA expression of the gene of the negative regulatory link of the peripheral molecular clock *cry1* ($P < 0.0003$), and among the genes of the positive link of regulation of the peripheral molecular clock *clock* and *bmal1*, there was noted a statistically significant increase in the evening expression of mRNA of the *bmal1* gene ($P < 0.0075$). Earlier, when analyzing the daily expression of circadian clock genes, it was demonstrated that the peak expression of the gene of the negative regulatory link of the peripheral molecular clock *per1* in the cells of the mucous membrane of the oral cavity was observed in the morning, and the highest expression of the *bmal1* gene was observed in the evening, the clock gene had no significant of daily fluctuations,[15] which coincides with the data we obtained regarding the expression of the *per1*, *bmal1* and *clock* genes. In addition, in our study, antiphase oscillations between *cry1* and *bmal1* genes were observed in the morning and evening, which was also investigated by Gu F. et al. (2021), who registered strong positive correlations in mRNA expression of *per* and *cry2* genes and negative correlations with *arntl* mRNA expression which indicates significant daily rhythms of gene expression in the tissues of the mucous membrane of the oral cavity in humans.[8]

However, the absence of a significant difference in *per1* gene expression may be associated with the presence of an inflammatory process in patients with maxillofacial Cellulitis. Thus, it was demonstrated that cytokines TNF-α and IL-1β might influence the circadian clocks resulting in the delay their phase oscillations.[16] Autoimmune and inflammatory processes directly affect the expression of *clock*, which can lead to a vicious cycle of increased inflammation.[17] It has been shown that the disruption of the circadian pattern of core clock gene expression leads to increased levels of pNFκB and IL-6, which demonstrates a shift of the PPAR-γ/NFκB/IL-6 axis in the pro-inflammatory direction.[18]

When dividing patients with odontogenic inflammatory diseases of the maxillofacial region into groups depending on chronotypes, our study showed that in patients with the morning chronotype, the evening expression of mRNA of the per1 gene was significantly lower than in the morning ($P < 0.006$).

Whereas in patients with the evening chronotype, the mRNA expression of the *per1* gene in the morning and evening did not differ significantly. At the same time, a probable increase in its evening expression was found in the group of patients with maxillofacial Cellulitis with the evening chronotype compared with patients who had the morning chronotype ($P < 0.006$), which may indicate a violation of the rhythmic expression of this gene in patients with the evening chronotype, which leads to the absence of its antiphase oscillation with the *bmal1* gene. Previously, when studying the expression of circadian genes over time between groups of chronotypes, regardless of gender and age of participants, it was demonstrated that the extreme evening chronotypes have a delayed phase of fluctuations in the *per3* and *nr1d2* circadian clock genes compared to the extreme morning types.[19,20]

It is possible that changes in the expression of the *per1* gene are one of the key factors in the development of inflammation and subsequent reparative processes in patients with maxillofacial cellulitis. It was discovered that changes in the expression of the circadian clock gene *per1* may play an important role in the occurrence and development of oral squamous cell carcinoma.[21] Studies on null genetic mouse models revealed impaired wound healing in some circadian mutants: NONO (RNA-binding protein, also called p54nrb, a member of the circadian transcription repressor complex in mice), *per1/2* and *bmal1* null mice, demonstrating the dependence of circadian gene activation of the cell cycle through NONO from the PER protein22. Recently, a more efficient course of reparative processes was demonstrated at the later stages of reparative regeneration in patients with Cellulitis of the maxillofacial area with a morning chronotype who were operated on in the morning.[23]

At the same time, it is known that people with an evening chronotype are more prone to the development of inconsistency of circadian clocks with the development of negative health consequences. It is shown that personality types D, characterized by negative affectivity and social inhibition with an evening chronotype, have a higher frequency of symptoms of temporomandibular disorders,[24] the evening chronotype in children is associated with a higher probability of developing allergic rhinitis and eczema,[25] a more severe course of non-alcoholic fatty liver disease is associated with an evening chronotype regardless of age, sex and body mass index.[26]

Therefore, in our study, a disturbance of rhythmic oscillations of the *per1* gene was observed in patients with cellulitis of the maxillofacial area, in patients with an evening chronotype compared to a morning chronotype, there were more significant manifestations of dysregulation of the circadian clock due to a probable increase in the expression of the *per1* gene in the evening and, as a result, the absence of its antiphase oscillation with the *bmal1* gene, which may affect wound healing processes after surgery and requires further study.

5. Conclusion

Thus, the obtained data indicate that in patients with odontogenic purulent inflammatory diseases of the maxillofacial area with an evening chronotype, a violation of the expression profile of the *per1* gene in the cells of the buccal epithelium is noted, which is manifested by an increase in its evening expression in comparison with patients with a morning chronotype. The significant increase in the level of *cry1* gene mRNA expression is found in the morning and the expression of *per1* gene mRNA in the morning and evening has not statistically significant changes during comparing morning and evening mRNA expression among the genes of the negative regulatory link of the peripheral molecular clock.

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Declaration of competing interest

All authors declare no conflict of interest.

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