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Original Article

Age-Related Features of the Pattern of Oral Fluid Patients with Non-Alcoholic Fatty Liver Disease

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Abstract

Introduction: In recent years, non-invasive screening methods for diagnosing various human conditions, including those corresponding to biological age, have attracted great interest, one of the sources for non-invasive research of which is oral fluid.

Aims: To study the age-related features of oral fluid facies in patients with non-alcoholic fatty liver disease.

Materials and methods: Sixty-one patients with non-alcoholic fatty liver disease and 57 somatically healthy patients were selected for study. An oral fluid sample was taken into a plastic tube in the quantity of 1.5-2 ml. The supernatant fluid was collected using a laboratory pipette, and 4 drops of 0.02 ml were formed on a glass slide. After that, the drop was subjected to natural dehydration. The facies study has been carried out using an electron microscope.

Results: Normally, during the phase OF drop transition, the formation of 2 zones is characteristic – the marginal zone having the "colloidal glass" form, and the central organized by crystals in the "fern leaf" or "tree branch" form. The metabolic dysfunction negatively affect the organization of protein molecules and the formation of marginal zone in which pathology markers are spread – fissures, wrinkles, pigment inclusions.

Conclusion: Accordingly, elements of crystal destruction appear in the salt part, and the number of chaotically oriented dendrites increases.

Keywords

aging, dehydration, facies, saliva drop

INTRODUCTION

Aging, as a physiological state, is the stage of any living organism's life cycle characterized by a decrease in the metabolic rate as well as in the level of protective and regenerative processes. All anatomical structures of the human body are also subject to aging physiology, the initiation of which is encoded at the gene level defining the "biological age" concept.[1-3]

A decrease in jawbone tissue density, a decrease in linear and space-occupying speed characteristics of regional blood flow, signs of mucous membrane atrophy, and changes in oral cavity sensory function – a reduction or rearrangement of food taste perception – as well as changes

in the qualitative and quantitative characteristics of mixed saliva are some of the changes that characterize the dentofacial system's organs and tissues during the physiological aging process.[4-6]

However, along with physiological aging, the initiation of premature aging of oral cavity organs and tissues is possible under the influence of a number of exogenous and/or endogenous risk factors: chronic stress, bad habits (smoking, abuse of sugary carbohydrate foods, non-compliance with the home oral hygiene algorithm), and somatic pathology, especially metabolic diseases. Under the influence of provocative factors, the stem cell pool is prematurely depleted, regenerative capabilities are reduced and the metabolism level changes contributing to the predominance of biological age over calendar one.[7]

In recent years, in medicine in general and dentistry in particular, non-invasive screening methods for diagnosing various human states, including compliance with biological age, have been of great interest. More often one of the diagnostic sources for non-invasive study is oral fluid (mixed saliva – OF), the morphological structure of which reflects both physiological and pathological body changes.[8-11]

OF consists of the discharge of all major and minor salivary glands, gingival fluid, food substrates, microflora and metabolites, desquamated epithelial cells, periodontal sulcus leukocytes, etc.[12] Under physiological, extremal and pathological body conditions, high-speed changes in the molecular composition and nature of the interaction of various components occur in the OF structure.[13] However, there is no possibility of observing and analyzing the OF liquid phase morphology, due to the high dynamics of changes in its structure per unit time. Study is possible only during the OF sample fixation process taking place at the moment of phase transition from liquid to solid state. OF drop drying contributes to phase transition and the fixed morphological structure is available for study and early diagnosis of both various pathological conditions and the premature aging process. To some extent, this can be defined as biological age micrograph.

OF drop preparation for study is called the wedge-shaped dehydration method that significantly expanded the possibilities of screening diagnostics. Oral fluid drop, located on a horizontal plane, functions as a convenient model of self-organizing system for studying physicochemical processes and makes it possible to identify patients' predisposition to the development of pathological conditions at the preclinical stage. During the phase transition process, a dry film – "facies" – is formed from the OF drop, which allows to stop the dynamics and fix unstable intermolecular bonds.[14]

It is known that during the mixed saliva drop drying, several zones are determined, which, according to various researchers, can be divided into 3 [central, intermediate and marginal (peripheral) zones], and into 2 zones [central (crystalline) and marginal (amorphous)].[15] The marginal-to-central zone ratio is important, where the latter should occupy at least 70% of facies area. This is the most favorable morphological pattern of facies corresponding to the optimal metabolism level during the normal life cycle course. It is also known that the central facies zone is formed from inorganic components organized into fern-like or tree-shaped crystals with straight course of the main branch from the periphery to the center, and the marginal zone is always transparent and solid (glass-like), without pathological markers. The 'wrinkles' in the marginal facies zone, integrity violation (fissures, ruptures), pigmentations, lumpy inclusions are considered manifestations of pathology and premature aging.

AIM

The aim of the present study was to study the age-related features of oral fluid facies in patients with non-alcoholic fatty liver disease.

MATERIALS AND METHODS

Characteristics of patients participating in the study

Sixty-one patients with metabolically associated diseases (non-alcoholic fatty liver disease), who were undergoing outpatient or inpatient treatment, were selected for the study. Underlying disease diagnosis was based on international recommendations and standards.[16] The majority of patients were men (75.4%). The control group consisted of 57 somatically healthy patients, representative with the main group by sex, nonsmoking, maintaining healthy diet, regularly visiting the dentist, and maintaining expedient oral hygiene. The patients in both groups were divided into age subgroups to identify expected differences and correspondence to their biological age **(Table 1)**.

Table 1. Distribution of patients by age groups

Age group (yrs)	Main group (n=61)	Control group (n=57)
18–29	6 (9.8%)	10 (17.5%)
30–49	19 (31.1%)	13 (23.0%)
50–59	28 (46.0%)	24 (42.0%)
60 and older	8 (13.1)	10 (17.5%)

In the main group, 22 (36.1%) patients smoked, carbohydrate foods predominated in the diet of most patients, and 43 (70.5%) did not maintain the regular oral hygiene regimen **(Table 2)**.

Oral fluid studies

For study purposes, an oral fluid sample was taken into a plastic tube in the quantity of 1.5-2 ml and centrifuged for

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Table 2. Division of the main group of patients

Age group	Smoking	Prevalence of carbohydrates in food	Regular 2-fold oral hygiene
18-29 years old	2 (33.3%)	4 (66.6%)	3 (50.0%)
30-49	11 (58.0%)	10 (52.6%)	7 (37.0%)
50-59	9 (32.1%)	19 (68.0%)	6 (21.4%)
60 and older	0	5 (62.5%)	2 (25.0%)

10 minutes at 6000 rev. The supernatant fluid has been collected using the laboratory disposable pipette, and 4 drops of 0.02 ml were formed on a glass slide. After that, the drop was subjected to natural dehydration at a temperature of 22-23°C, at a relative humidity of 60%–70%, the drying period was 18–24 hours. During the dehydration process, a phase transition took place with the OF facies formation. The facies study was carried out using a Micros electron microscope with magnification of 40×–100×. The images were entered into the computer via the standard USB interface using video attachment with software and image capture. Observations were carried out in "light" and "dark" fields, both in ordinary and polarized light. The analysis of facies zoning, central zone crystallization and marginal zone integrity, presence of pathology markers in the form of pigmentation, fissures, wrinkles, lumpy and inclusions, was carried out.

Statistical analysis

Statistical analysis was carried out using SPSS. The mean M value, standard m error, and Student's test were used to compare features. Comparison of percentages (proportions) was carried out using the z-test. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

When studying the control group OF to identify morphological age differences, we established that the facies of the group of somatically healthy patients under 29 years of age had a clear division into zones with central zone predominance. The mineral components in the central zone were organized in the form of crystals having the straight course of main branches from the periphery to the center located at an acute angle to each other. The branches of the 1st and 2nd orders with rounded ends gave them a fern-like shape. In the facies center, destruction of crystals in the form of small dendrites-fragments was not observed. The marginal zone formed by organic components had the "colloidal glass" appearance and evenly surrounded the salt zone **(Fig. 1)**. There were no pathological markers.

When analyzing the facies of the patients aged 30-49 years, a clear division into zones was also observed, while the central (salt) size predominated over the peripheral (colloid). In this age group, we observed the appearance of small radial fissures in the marginal zone and single small ruptures along its outer contour. In the salt zone, the crystals retained the fern leaf configuration, but were smaller in size, and chaotically oriented dendrites were found closer to the center **(Fig. 2a)**.

For patients of the third age subgroup (50-59 years), the appearance of wrinkling and curved fissure areas in the marginal facies zone became characteristic. Meanwhile, the marginal zone covered the facies perimeter with uniform colloidal rim and did not have any pigmented inclusions. In the central zone, there were separate straight crystals with bilateral 1st order branches running from the periphery to the center, but dendrites of different sizes and shapes with chaotic localization over the entire area of the salt zone predominated in a greater degree indicating the

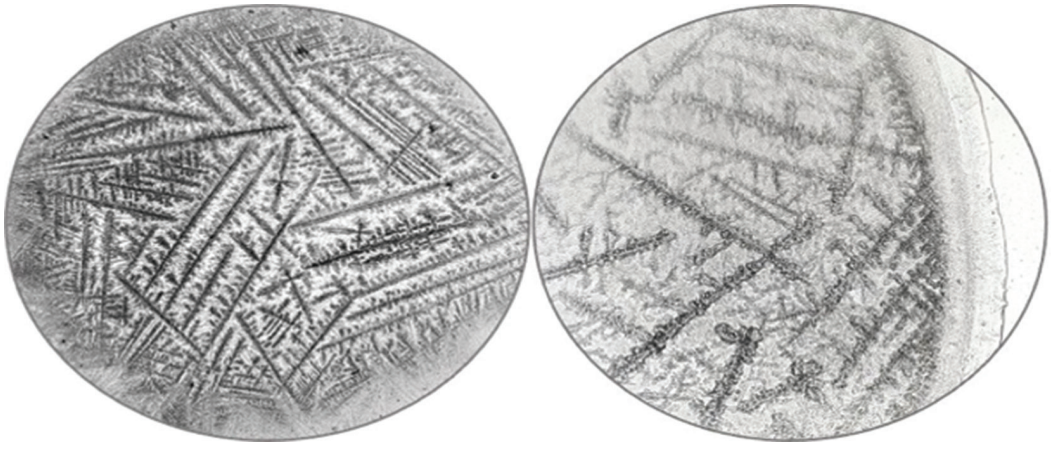

Figure 1. Facies micrograph of the 1st age subgroup control patient.

additional crystallization centers. The general configuration of crystals in the central zone resembled the "frost patterned glass," different from the two previous age subgroups **(Fig. 2b)**.

The oldest age subgroup (above 60 years of age) was distinguished by "wrinkles" with rupture and fissure areas along the entire plane of marginal zone, which, however, retained the colloidal glass appearance and was equidimensional along the entire facies circumference. Most of the salt zone crystals had straight course of the main trunks from the periphery to the center at an angle to each other, but they were much thinner, and the branches of the 1st and 2nd orders more often had one-sided localization. Also, there were large-sized chaotic dendrites localized closer to the facies center. In general, the pattern of salt zone crystals had tree-like nature being the variant of norm for these patients **(Fig. 2c)**.

The somatic status feature of patients in the main group was the metabolically associated pathology, the pathogenesis of which is based on insulin resistance and low-intensity systemic inflammation. This endogenous factor will influence the dynamics in the OF processes manifesting itself during the phase transition into facies. In addition, as pointed out above, more than half of the main group patients abused carbohydrate foods and did not maintain the home oral hygiene algorithm, which amounted to 62.2% and 70.5%, respectively. More than a third, namely 36.1% of the total main group number, smoked and the listed bad habits were visualized in the OF facies.

When analyzing the facies of young patients (under 29 years of age) from the main group, the marginal zone had the colloidal glass appearance, uniformly surrounding the salt part, without pathological markers. However, between the marginal and central zones, the mineral component concentration visualized in the form of a ring, from which thin straight crystals grew, having small branches of the 1st and 2nd orders with sharp ends. Most of the salt zone crystals were represented by chaotically oriented dendrites of various sizes and shapes and dark pigment inclusions localized in the facies center. The general central zone pattern only in places resembled a "fern leaf."

In the presence of bad habits (smoking and abuse of carbohydrate foods), the facies of main group patients under 29 years of age had a decrease in the salt zone area, the crystals looked like thin threads with thickening towards the center and numerous pigment inclusions. There were many small chaotic dendrites throughout the entire central zone area. Th e general central zone background was represented by dark pigment spot. The marginal zone did not have the colloidal glass appearance, was unevenly expanded, contained wrinkling areas along the perimeter and multiple pigmented lumpy inclusions along the entire width **(Figs 3a, 3b)**.

The main group patients from 30 to 49 years old had different facies pattern characterized by wide intermediate zone, due to the thickening of mineral salts, with pigment inclusions – lumps and spots. The crystals began as thin trunks, gradually widening towards the center, with predominantly one-sided branches with sharp ends giving the appearance of a "comb" pattern. In the center there were many small dendrites in the "star and crosses" form **(Fig. 3c)**. Also, pigment inclusions occurred throughout the salt zone area, especially in the center.

Multiple pigment spots, inclusions resembling a "tufts-of-grass" pattern, and arched fissures in the marginal zone were characteristic of the 3rd age subgroup patients (50-59 years of age). The central zone crystals were straight without branches, resembled a "spaghetti" pattern, and there were thin chaotic dendrites. The central zone of this age category was characterized by pigment inclusions in the form of sticky conglomerates **(Fig. 4a)**. In addition, there were facies in which thin small crystals formed only along the salt zone perimeter, and its central part was filled with pigmented "pieces of mica" inclusions typical for manifestation of inflammatory and destructive processes.

Patients from this age subgroup, but with past medical history of bad smoking habit, were characterized by a different morphological OF facies pattern. They were

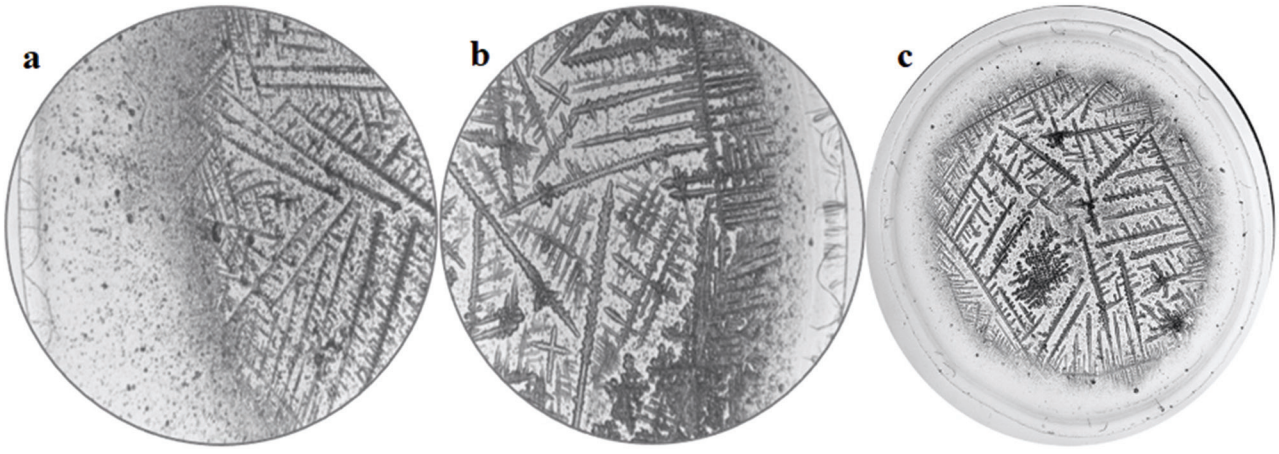

Figure 2. (**a**) Facies micrograph of the 2nd age subgroup control patient; (**b**) facies micrograph of the 3rd age subgroup control patient; (**c**) facies micrograph of the 4th age subgroup (above 60 years of age).

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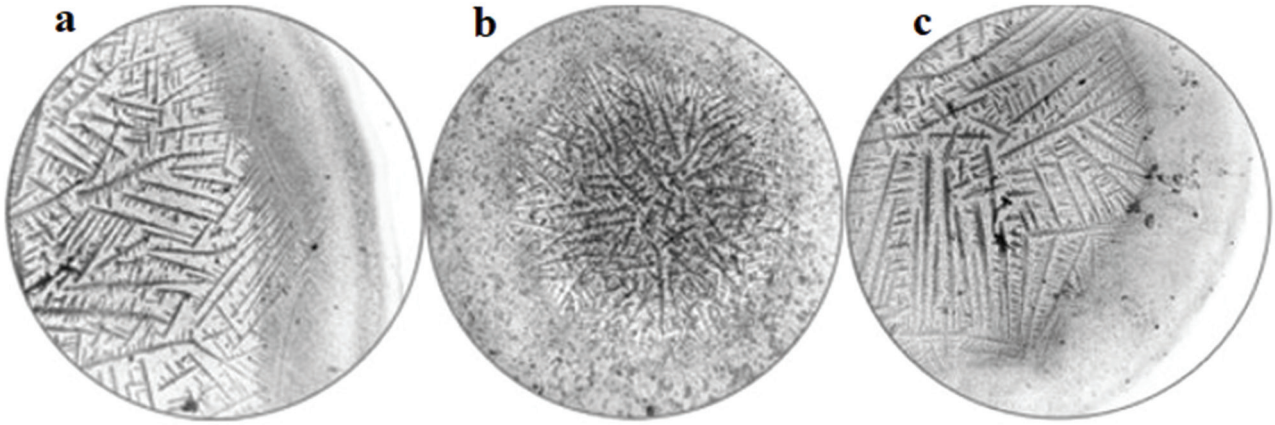

Figure 3. (**a**,**b**) Micrograph of a 34-year-old patient of the main group; (**c**) Micrograph of the main group 30-49 years old patient.

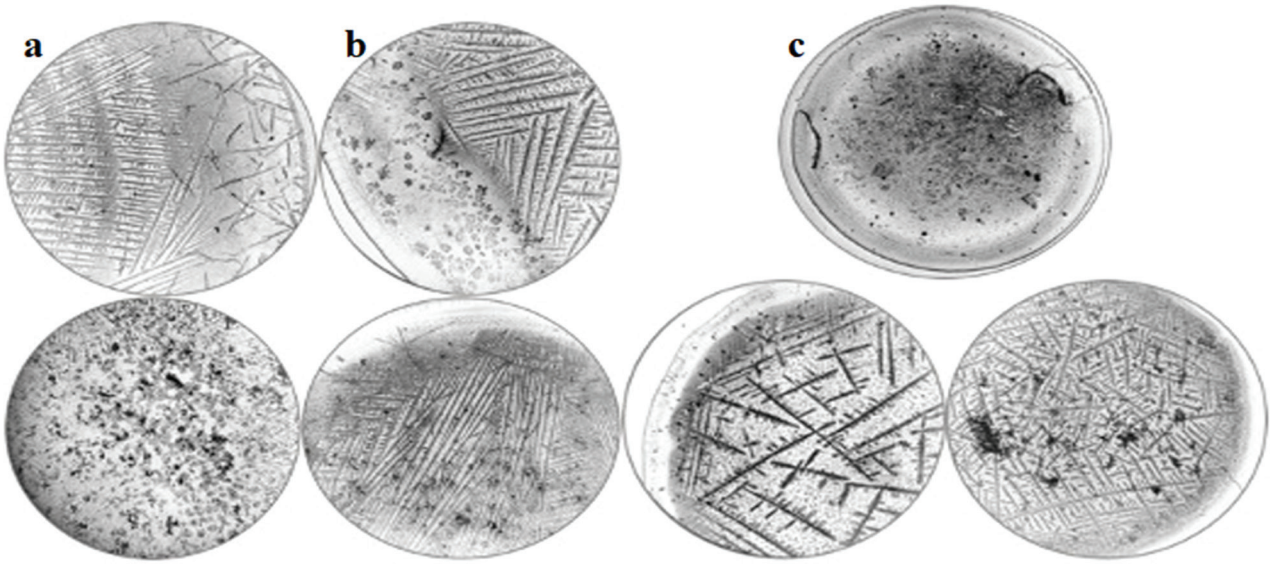

Figure 4. (**a**) Micrograph of the main group patients (subgroup of 50-59 years of age); (**b**) Micrograph of facies of a 50-year-old smoker from the main group; (**c**) Micrograph of the main group patients above 60 years of age.

characterized by the absence of boundary between the zones, the salt zone was displaced from the facies center and had the pigmented conglomerate appearance without characteristic crystallization **(Fig. 4b)**. The marginal zone did not have a "colloidal glass" appearance, it contained pigmented lumps and other inclusions, as well as deep curved ruptures reaching into the area of mineral facies components.

The central zone of the main group patients above 60 years of age was represented by thin chaotically oriented dendritic crystals of different sizes and shapes with pigment inclusions in the form of sticky conglomerates, or the crystals themselves were dark. There were facies where the density of central zone filling with crystals was low. There was the ring area of mineral component concentration along the salt zone perimeter. Multiple fissures, ruptures and pigment inclusions were observed in the marginal zone **(Fig. 4c)**.

DISCUSSION

The phase OF drop transition into the solid structure-facies takes place with alternation of certain physicochemical processes over time. First of all, free water evaporates leading to gel formation. Protein molecules, in a greater degree, due to centrifugal oncotic forces, are concentrated along the edge, creating the edge zone, but some organic components form the spatial lattice for subsequent crystallization.[17,18] Due to the drop shape, water evaporation primarily occurs along the edge with gradual shift towards the center triggering osmotic centripetal currents. The concentration area of mineral salts on the inner marginal zone boundary is the crystallization center, the completion of which ensures the formation of characteristic configuration in the "fern leaf" form.

In somatically healthy patients without bad habits main-

taining oral hygiene algorithm, there is the central (salt) zone predominance over the marginal (organic) along the facies area.[19] The crystallization process takes place from the periphery to the center, while the main trunks are located at an acute angle to each other and have branches of the 1st and 2nd orders with expanded rounded ends. The formed crystals form a "fern leaf" or "tree branch" pattern. With age, the characteristic feature is an increase in the number of dendritic crystals localized chaotically throughout the salt zone indicating the appearance of additional crystallization centers. Such combination of crystals can form a "frost glass" pattern. In the older age subgroup, the size and number of straight crystals decrease, and the number of centers of additional crystallization increases, but the tree-like pattern nature remains. The marginal zone retains the "colloidal glass" structure in all age subgroups and uniformly surrounds the salt facies part. The characteristic feature, with aging, is the appearance of separate wrinkling areas with gradual spread of the "wrinkle" pattern over the entire marginal zone area. Also, small fissures have been observed quantitatively increasing in direct proportion to age and qualitatively acquiring an arched or intertwined appearance. Small ruptures, limited by outer marginal zone perimeter, deepened with age, curved and could reach the crystallization centers.

With concomitant metabolically associated diseases, metabolic body disorders occur, affecting the difference between calendar and biological age in favor of the latter. Perhaps the spatial structure of salivary proteins or the sequence of their dipole molecules changes leading to other intermolecular interactions with water and mineral salts. Accordingly, various aging markers appear earlier in the marginal zone in the form of multiple fissures, wrinkles and ruptures, the number of chaotically oriented crystals increases and their sizes decrease in the central zone.

During the phase OF drop transition process, intermolecular interactions are very sensitive to various impurities, the inflow of which is ensured by bad habits. Impurities contribute to the appearance of additional crystallization centers in the central zone promoting the growth of chaotic crystals of different sizes and shapes. In addition, for example, the smoking saturates saliva with negative components manifested by increase in pigment inclusions over the entire facies area, and failure to maintain home oral hygiene contributes to the appearance of pigment conglomerates and darkening of crystals. Chronic inflammation also has destructive component manifested by increase in the number of small crystals in the cross or star form, the appearance of fragments and decrystallization area in the facies center filled with various inclusions.[20]

CONCLUSION

Normally, during the phase OF drop transition, the formation of two zones is characteristic – the marginal (protein) zone having the "colloidal glass" form, and the central (salt) organized by crystals in the "fern leaf" or "tree branch" form. The central zone predominates over the marginal zone, which, in turn, evenly surrounds the crystals.

The orderliness of facies structure begins with organization of protein molecules forming both the marginal zone and the organic grid-frame for subsequent crystallization of salts. Namely, the normal organization of protein molecules is determined by metabolism level and characterizes biological age.

The metabolic dysfunction negatively affect the organization of protein molecules and the formation of marginal zone in which pathology markers are spread – multiple fissures, wrinkles and ruptures, and pigment inclusions. Accordingly, elements of crystal destruction appear in the salt part, and the number of chaotically oriented dendrites increases.

Bad habits contribute to an increase in the number of various OF impurities, to which their components are sensitive at the time of phase transition with characteristic facies pattern formation where biological age predominates over the calendar one, especially in patients with metabolic diseases, in particular, the non-alcoholic fatty liver disease.

An integrated approach with effective minimally invasive studies of oral fluid facies is necessary for the development and promotion of new diagnostic and therapeutic and preventive methods for the correction of premature aging in patients with non-alcoholic fatty liver diseases (NAFLD).

Further studies are needed to identify prognostic factors in the oral fluid of patients with NAFLD as early markers that influence the rate of onset of aging in different age groups.

Conflict of interest

The authors declare that there is no conflict of interest in relation to this paper and the published research results.

Declaration of funding

This study was not sponsored.

Data availability statement

Data will be made available on reasonable request.

Use of artificial intelligence

The authors attest that they did not create the current work using artificial intelligence technologies.

Ethics statement

All participants have been provided with information to sign an informed consent for participation in the study. The measures provided to ensure safety for the patient's health, respect for his/her rights, human dignity and moral-and-ethical norm comply with the principles of the Helsinki Declaration of Human Rights, the Council of Europe Convention on Human Rights and Biomedicine, the Council for International Organizations of Medical Sciences, the International Code of Medical Ethics and relevant legal acts regulating the conduct of clinical studies.

Author contributions

Conception: N.E., D.E.; design: D.E.; supervision: N.E.; funding: D.E.; materials: D.E.; data collection and/or processing: N.E.; analysis and/or interpretation: N.E., D.E.; literature search;:D.E.; writing: N.E.; critical review: N.E.

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Возрастные особенности структуры ротовой жидкости у больных неалкогольной жировой болезнью печени

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Резюме

Введение: В последние годы большой интерес вызывают неинвазивные скрининговые методы диагностики различных состояний человека, в том числе соответствующих биологическому возрасту, одним из источников неинвазивного исследования которых является ротовая жидкость.

Цели: Изучить возрастные особенности фаций ротовой жидкости у больных неалкогольной жировой болезнью печени.

Материалы и методы: Для исследования были отобраны 61 пациент с неалкогольной жировой болезнью печени и 57 соматически здоровых пациентов. Проба ротовой жидкости отбиралась в пластиковую пробирку в количестве 1.5-2 ml. Надосадочную жидкость отбирали с помощью лабораторной пипетки и формировали на предметном стекле 4 капли по 0.02 ml. После этого каплю подвергали естественному обезвоживанию. Исследование фаций проводили с помощью электронного микроскопа.

Результаты: В норме во время перехода фазы в каплю характерно образование 2 зон – краевой, имеющей форму «коллоидного стекла», и центральной, организованной кристаллами в форме «листов папоротника» или «ветки дерева». Нарушение метаболизма негативно влияет на организацию белковых молекул и формирование краевой зоны, в которой распространяются маркеры патологии – трещины, морщины, пигментные включения.

Заключение: Соответственно, в солевой части появляются элементы разрушения кристаллов, увеличивается количество хаотично ориентированных дендритов.

Ключевые слова

старение, обезвоживание, лицо, капля слюны