Results in Physics 6 (2016) 315–321

Contents lists available at ScienceDirect

Results in Physics

journal homepage: www.journals.elsevier.com/results-in-physics

The investigations of changes in mineral–organic and carbon–phosphate ratios in the mixed saliva by synchrotron infrared spectroscopy

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ARTICLE INFO

Article history:
Received 27 May 2016
Accepted 7 June 2016
Available online 11 June 2016

Keywords:
IR spectromicroscopy
The remineralisation of tooth enamel
Caries prevention

ABSTRACT

The objective of this study was to investigate the efficiency of the saturation of mixed saliva by mineral complexes and groups necessary for the remineralisation of tooth enamel using exogenous and endogenous methods of caries prevention.

Using IR spectroscopy and high-intensity synchrotron radiation, changes in the composition of the human mixed saliva were identified when exogenous and endogenous methods of caries prevention are employed. Based on the calculations of mineral/organic and carbon/phosphate ratios, changes in the composition of the human mixed saliva depending on a certain type of prevention were identified.

It is shown that the use of a toothpaste (exogenous prevention) alone based on a multi-mineral complex including calcium glycerophosphate provides only a short-term effect of saturating the oral cavity with mineral complexes and groups. Rinsing of the oral cavity with water following the preventive use of a toothpaste completely removes the effect of the saturation of the mixed saliva with mineral groups and complexes.

The use of tablets of a multi-mineral complex with calcium glycerophosphate (endogenous prevention) in combination with exogenous prevention causes an average increase of ~10% in the content of mineral groups and complexes in the mixed saliva and allows long-term saturation of the oral fluid by them. This method outperforms the exogenous one owing to a long-term effect of optimal concentrations of endogenous and biologically available derivatives of phosphates on the enamel surface.

Introduction

Cariogenic pathologies affect health and physical appearance, and thus social and professional activities. The susceptibility to caries is related to the structure and properties of the dental tissue, the structure of dentitions and jaws, diet, the composition of the mixed saliva, the quality of the performed dental prophylaxis and the level of health of the person as a whole [1–4].

It is well-known that resistance to caries is directly dependent on the composition of the mixed saliva containing organic components as well as inorganic ions coming both from salivary glands and from outside [5–9].

Modern research into caries prevention tends to make use of the general foundations of caries prevention. The first one is regular hygiene of the oral cavity using a variety of exogenous methods: brushing teeth using a toothpaste, dental floss, rinsing and application of special prevention tools. This not only provides mechanical cleansing of the tooth surface but also mineral substances from the exogenous tools into the oral cavity. It is thought that if exogenous prevention is duly provided, there is nothing stopping normal organic and mineral exchange from taking place between the oral cavity and enamel to restore the latter [5,10].

The second foundation is based on maintaining the microelement, organic and mineral composition of the mixed saliva so that the enamel restores naturally [11]. This becomes possible owing to the mineral components of medications, i.e., endogenous prevention. It should be noted that endogenous prevention is used in combination with exogenous prevention.

No or insufficient prevention causes caries. This kind of disease takes place under the accumulation of numerous cycles, including the processes of demineralisation and remineralisation, and takes from a month to a year [1,12,13]. The balance in the cycles is shifted towards the first of the processes (demineralisation) as a result of the vital activity of bacteria, removal of some part of the biofilm from the surface of enamel, peracidity, inadequate

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http://dx.doi.org/10.1016/j.rinp.2016.06.005
2211-3797/

organic-mineral composition of the mixed saliva and the absence of necessary preventive measures [1,2,14–16]. It has been experimentally proven that development of demineralisation is not a unidirectional destruction but rather a periodical process. It means that demineralisation in the form of destruction/dissolution of the structure motifs of enamel (nanocrystals of calcium hydroxyapatite (HAP) and its substituted forms) as a result of mechanical impact or bacteria activity [17,18] is interchanged with the stages of recovery of the hard dental tissues [4,13,19,20]. The one-time process of dental enamel recovery inverse to the demineralisation is generally named as remineralisation and is realised owing to the mixed saliva being oversaturated with ions (Ca^{2+}, HPO_4^{2-}, Na^+, Mg^{2+}, Cl^-, F^-, OH^-, etc.) relative to the dental enamel. The essence of remineralisation is in the saturation of the upper layers of enamel with these ions and complexes, their further crystallisation, and formation of the lost mineral forms and motifs in the affected areas [21,22].

However, despite numerous approaches to finding more effective caries prevention methods [2,23,24], the interaction between the employed prevention methods, the composition of the mixed saliva and the efficiency of the saturation of the mixed saliva with ions and complexes to make remineralisation possible have not been thoroughly studied.

As was previously noted, remineralisation can be triggered artificially. This is called "remineralising therapy". It is based on the possibility of a purposeful change and maintaining the mixed saliva state (its acidity, mineral and organic composition) at a certain level when a human organism can supply all of the mineral losses [24–28]. With experience of the application and comparison of different prevention methods, including fluoridation, it is possible to ensure that the use of specimens involving phosphorus- and calcium-containing agents provides the best results in the remineralisation of dental enamel [29–35]. This in turn allows one to argue that the absence of these ions in the mixed saliva causes normalisation of exchange processes in the oral cavity. Studies of prophylaxis efficiency performed by the exogenous methods, namely tooth brushing and gel application, have demonstrated the efficacy of this trend and the character of activity [32]. However, we think that exogenous methods of prevention do not guarantee constant maintenance of the level and saturation of the mixed saliva by mineral and organic ions and complexes.

In natural conditions, mixed saliva is saturated with phosphates and calcium ions; thus, in the nearest environment of enamel prisms (the mineral component of the enamel is hydroxyapatite) there is a sufficient amount of the ions Ca^{2+}, HPO_4^{2-}, F^- and OH^-. One should note that remineralisation of enamel is not very efficient at high concentrations of calcium ions and phosphates in the saliva or artificial calcium-containing tools [5] as these conditions cause the formation of a dental calculus. Optimal remineralisation of the caries region and artificially stimulated caries-like lesions of enamel is realised under low concentrations of calcium ions and phosphates (1–2 mmol/l), while in the demineralised region within the near-surface region of enamel these values are about 0.1 mmol/l [5].

In order to meet these conditions, remineralising agents should not only be maintained in necessary concentrations in the oral fluid but also be kept in the oral cavity for a considerable time and come in contact with the dental enamel. As was previously pointed out, a necessary condition for remineralisation is ionised mineral substances in the oral fluid, which is not very likely in exogenous ion introduction. We believe that endogenous and biologically available phosphorus and calcium derivatives can not only saturate the oral fluid with necessary mineral ions but also enhance remineralisation as there can be conditions where they can be kept in the oral fluid.

Therefore, in order to saturate the oral fluid with calcium ions and phosphorous derivatives it is promising to use calcium glycerophosphate as an active component of different preventive measures [36,37]. Different articles dealing with remineralisation of the dental enamel make an assumption about the efficiency of the use of calcium glycerophosphate [36–40]. We believe the use of endogenous prevention with calcium glycerophosphate to be capable of enhancing the saturation of the oral fluid with calcium and phosphate ions.

However, there have been no studies to identify changes in the composition of the oral fluid, its saturation with mineral ions and complexes, or the longevity of the ions enabling remineralisation of the dental enamel of the complexes if exogenous and endogenous caries prevention has not taken place. What has been established is only that prevention and remineralisation levels interact or depend on each other in ways that can be described.

Hence, the objective of our study is to investigate the efficiency of using exogenous (use of a toothpaste) and endogenous (a pelleted mineral complex based on calcium glycerophosphate) methods of caries prevention to saturate the oral fluid with mineral complexes and groups necessary for remineralisation of the dental enamel.

Materials and methods

Two hundred people (100 men and 100 women) from the age of 22–30, healthy, addiction-free, and university-educated took part in the study. All of them provided their own mixed saliva for the investigations.

During the experiment and a week prior, the patients mainly ate vegetable food, followed a standard water consumption pattern, did not take any remedies, and consumed no alcohol.

The regulations of making the mixed saliva sample were as follows (see Fig. 1).

Seven days later (after the beginning of the experiment without the changes of conditions for hygienic measures of the oral cavity), in the morning before eating, their first meal participants of the experiment sampled their own mixed saliva for the first time after oral rinsing with water. On the same day after the collection of a mixed saliva sample, the patients used the same toothpaste to brush their teeth. Five minutes later (after proceeding with the hygienic measures for the oral cavity with the use of a toothpaste and an oral rinsing with water), mixed saliva was sampled once again. Thirty minutes later (and once again after an oral rinsing for 30 s), the mixed saliva was sampled by the patients for the third time.

On the next day, the participants of the experiment began to take the same tablets (a pelleted mineral complex on the basis of calcium glycerophosphate). Participants of the group took one tablet three times a day. Three days after that in the morning, on an empty stomach, the patients thoroughly carried out oral rinsing with water and they were again subjected to mixed saliva sampling.

Each time after the sampling mixed saliva was immediately centrifuged in order to remove excess water. The residue was dried at 36 °C in a desiccator.

It should be noted that in this research we have investigated mixed saliva (oral fluid) with the composition being different from that of a mix of salivary glands. For exogenous and endogenous prevention there will be derivatives of the used prevention substances in the mixed saliva which will enter it either through the oral cavity (a toothpaste) or through salivary glands (use of a mineral complex based on calcium glycerophosphate).

Investigations of the samples obtained in such a way were performed by IR spectroscopy, which is widely used for the analysis of

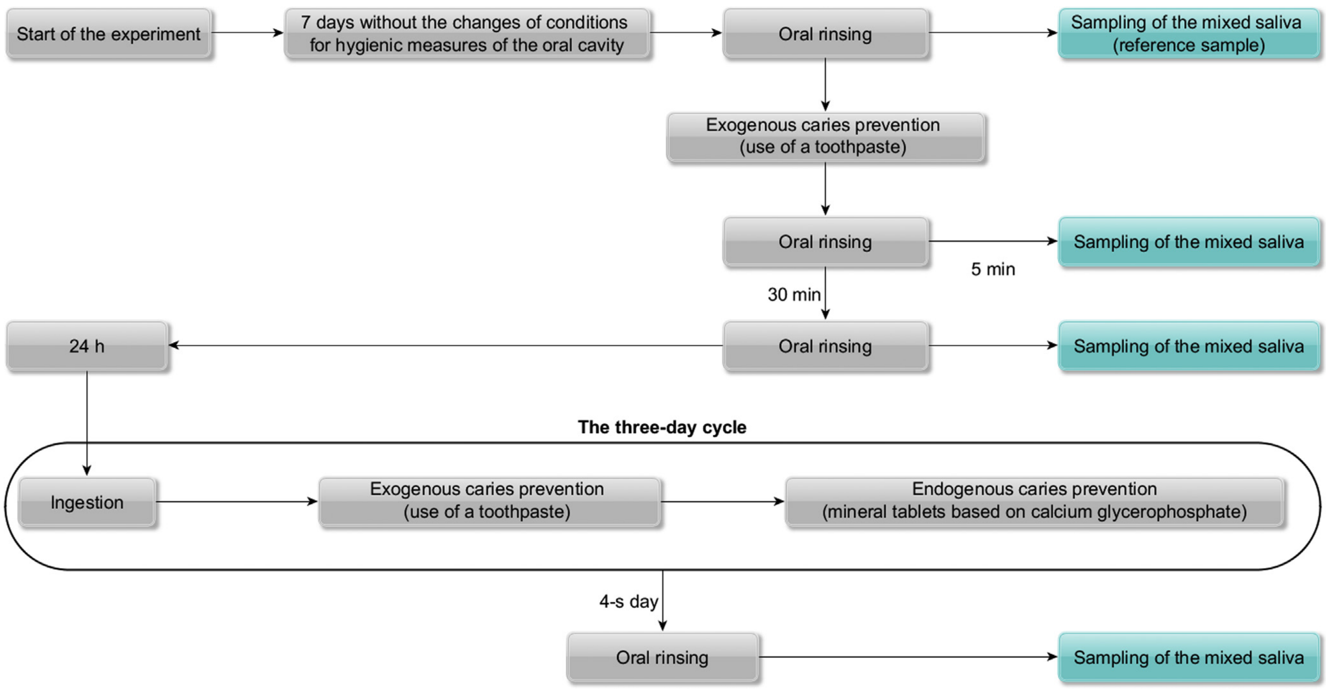

Fig. 1. The regulations of making the oral fluid sample.

dental materials and for the detection of the nature of pathologies in the extracted teeth [41–43].

As for the dental applications, the advantage of this method is its high selectivity and sensitivity. It allows one to obtain extensive and various information on the structure of substances, to consider the effect of atomic groups comprising compounds, as well as to detect neogenic mineral phases. One more advantage of IR spectroscopy is the possibility to apply it in the analysis of the multi-component compounds. Taking into account the fact that the main difficulties in the study of organic compounds are usually referred by their identification and estimation, IR spectroscopy is actively applied for the determination of the qualitative and quantitative composition of the dental samples and analysis of the changes proceeding in the human mixed saliva under different impacts. Unlike a lot of the other methods, the investigated system is subjected to a very weak external effect when using this technique. Thus, the obtained information is related to the system that did not undergo any changes as a result of its interaction with IR radiation.

The investigations of the molecular structure of the mixed saliva and its chemical composition after the described sampling of specimens were performed utilising the equipment of the Infrared Microspectroscopy (IRM) beamline at Australian Synchrotron, Victoria, Australia. A Bruker V80v Fourier transform infrared (FTIR) spectrometer and a Hyperion 3000 IR microscope (Bruker, Germany) were used to perform the analysis of a microprobe of the specimens as well as a diamond high-pressure cell. Transmission IR spectra were measured within the range of 4000–500 cm^{-1}.

Experimental results

With the use of super-modern equipment for IR spectromicroscopy and high-intensive synchrotron radiation with an excellent beam collimation, IR transmission spectra of the dry residues of mixed saliva collected at different stages of the experiment (according to the described regulations) were obtained for two groups of participants. The obtained spectra are presented in Figs. 2 and 3.

As is seen from these figures, the application of synchrotron radiation for the analysis of samples (together with the IR-microscope) it possible to obtain IR transmission spectra of the samples, providing a better signal-to-noise ratio and a better resolution.

A primary analysis of all of the experimental data demonstrated that all of the obtained spectra include just the same set of vibration modes and were only slightly different in the intensities of certain vibration modes.

Taking into account this fact, only normalised IR transmission spectra of the dry residues of the mixed saliva were averaged over the group of patients participating in the experiment (as presented in Figs. 2 and 3).

Fig. 3 represents IR transmission spectra obtained with the use of synchrotron radiation and a high-pressure attachment with a diamond prism to an IR microscope: (1) IR spectrum of the mixed saliva taken before the experiment (spectrum of the reference sample), (2) IR spectrum of the mixed saliva taken on the fourth day after a three-day taking of the tablets on the basis of calcium glycerophosphate for three days and oral rinsing, (3) spectrum of the tablet on the basis of calcium glycerophosphate.

The analysis of the obtained spectra was performed based on a number of data taken from a set of reference sources, where mixed saliva, hard dental tissues of a human, as well as phosphates related to the formation of dentin and enamel were investigated by means of IR spectroscopy [44–47]. A list of the active vibrations in the spectra of the first and second stages of the experiments, the ranges, and the frequencies with the peaks of vibration bands are presented in Table 1 along with their assignments to the groups of vibrations.

From the obtained experimental data (Figs. 2 and 3, Table 1), it follows that the main vibration bands in the IR transmission spectra of all of the samples representing dry residues of the mixed saliva obtained in the experiment can be assigned to the following groups and complexes. The first and most interesting group of high-intensity vibrations arranged in all spectra within 900–1200 cm^{-1} is related to the modes, and their appearance is con-

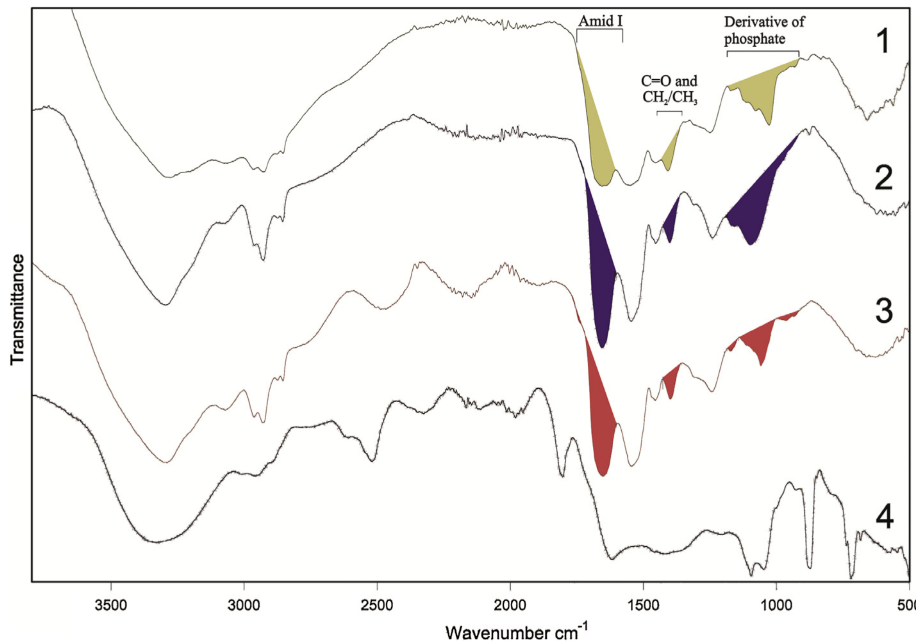

Fig. 2. IR spectra obtained of the mixed saliva taken: (1) IR spectra of the mixed saliva taken before the experiment (the reference sample), (2) spectra of the mixed saliva taken off the patients five minutes after the use of a toothpaste and an oral rinsing, (3) spectrum of the mixed saliva taken 30 min after the use of a toothpaste and an oral rinsing, (4) spectrum of the used toothpaste with a multi-mineral complex on the basis of a calcium glycerophosphate.

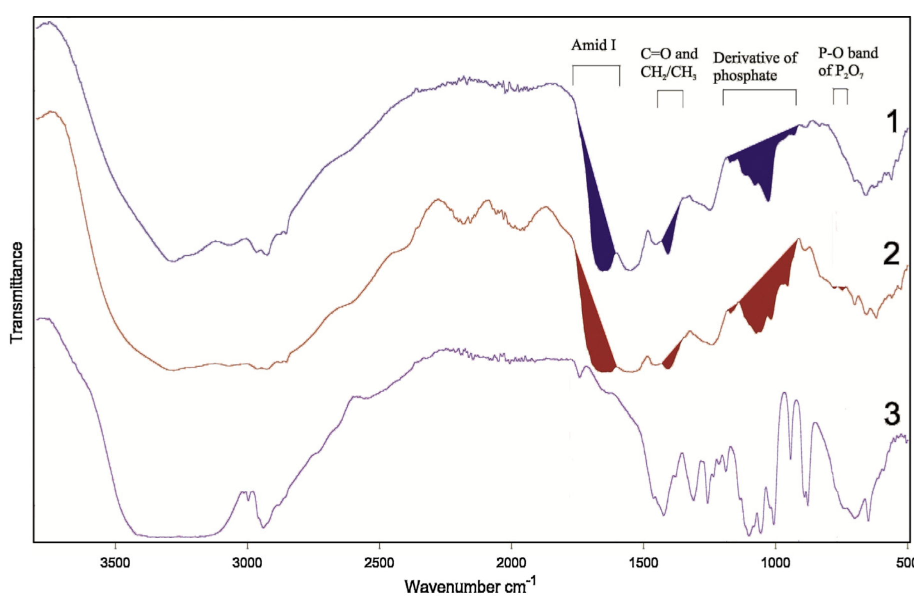

Fig. 3. IR spectra of the mixed saliva taken before and after the use of the tablets (1) IR spectra of the mixed saliva taken before the experiment (spectra of the reference sample), (2) IR spectrum of the mixed saliva taken on the fourth day after a three-day taking of the tablets on the basis of calcium glycerophosphate and an oral rinsing, (3) spectrum of the tablet on the basis of calcium glycerophosphate.

nected with the presence of phosphorus derivatives in the samples, such as phosphates, glycerophosphates and phospholipids. This conclusion can be made by comparing the data presented in [48], where a detailed analysis of the spectra of mixed saliva and human blood was performed.

The next big group of vibration bands localised in the range of 1240 cm^{-1}–1700 cm^{-1} can be related to the secondary amides: Amid I, Amid II and Amid III. Modes arranged in the experimental spectra in the range of 1400–1430 cm^{-1} belong to C=O stretch (sym) vibrations of COO and CH_2/CH_3 groups.

One should note that in the IR spectra of the mixed saliva collected on the fourth day, after three-day taking of the tablets on the basis of a calcium glycerophosphate and the spectrum of the tablet on the basis of calcium glycerophosphate, there is one more additional group of vibrations with a peak in the range of 730–770 cm^{-1}. Based on the principles described in [47], it seems impossible to correlate a group of vibrations characterised by the maxima at 730–770 cm^{-1} with the vibrations of C–H bonds. However, this vibration mode can be related to the vibrations of the P–O bond in the P_2O_7 group. The arising of these vibration modes was already observed in [49] under thermal decomposition of the inorganic compounds.

Figs. 2 and 3 represent IR spectra of the mixed saliva samples (together with the transmission spectra of a toothpaste with a

Table 1
Active vibration bands in the IR spectra of mixed saliva in the experiments and reference data.

Substance	Vibration modes	Wavenumbers cm^{-1}	References
Phosphates	P–O band of P_2O_7	735–775	[47]
	Oligo, polysaccharides, and phosphorus derivatives	1025–1080	[48]
	Derivative of phosphate, glycerophosphate and phosphatase. Phospholipids	1055	[48]
Proteins (α-amylase; albumin; cystatins; mucins; proline-rich proteins; sIgA) -Hormones	P=O str (asym) of >PO_2–phosphodiesters Stretching vibrations of C=O groups (Amide I)	1240–1245	[44,45]
	Amide III (CN stretching, NH bending) band components of proteins	1270	[44]
	stretch – carboxyl group vCOO	1330–1345	[44,45]
	C=O str (sym) of COO^-	1400	[44]
	CH_2/CH_3	1395–1410, 1450	[44,45]
	Amide II (CN stretching, NH bending)	1545–1555	[44,45]
	(HNH)(NH_2) Amide II (CN stretching, NH bending vibrations)	1570	[45,45]
	Amide I (C=O stretching) of β-pleated sheet structures Amide I (C=O stretching) and (COO-) stretching vibration	1645–1650	[44,45]
Lipids (cholesterol and mono/diglycerides of fatty acids)	C–H str (sym) of – CH_3	2875	[44]
	C–H str of C–H in methine groups	2900	[44]
	C–H str (sym) of >CH_2 in fatty acids	2925	[44]
	C–H str (sym) of >CH_2 in fatty acids	2965	[44]
Proteins (α-amylase; albumin; cystatins; mucins; proline-rich	Primary and secondary amines (H_2 and NHR)	3065	[44]
proteins; sIgA)	N–H str (amide A) of proteins	3205	[44]
-Hormones (cortisol; testosterone)	N–H str (amide A) of proteins	3290–3295	[46]
Proteins	Amide IV OCN bending of proteins	625–765	[47]
	Amide V Out-of-plane NH bending of proteins	640–800	[47]
	Amide VI Out-of-plane C=O bending of proteins	535–605	[47]

Table 2
The results of calculations of relative changes in mineral–organic and carbon–phosphate ratios in the mixed saliva in comparison of the reference sample at the different stages of the experiment.

The stages of the experiment	Relative change in mineral–organic ratio, %	Relative change in carbon–phosphate ratio, %
5 min after the usage of a toothpaste involving the multi-mineral complex on the basis of calcium glycerophosphate + oral rinsing (exogenous prevention)	+7.3 ± 2.3	–9.4 ± 2.7
30 min after the usage of a toothpaste involving the multi-mineral complex on the basis of calcium glycerophosphate + oral rinsing (exogenous prevention)	–1.8 ± 2.0	–2.0 ± 1.8
On the fourth day after taking of the tablets (pelleted multi-mineral complex on the basis of calcium glycerophosphate) + oral rinsing (endogenous prevention)	+11.8 ± 1.9	–53.7 ± 1.5

multi-mineral complex) on the basis of calcium glycerophosphate and IR spectra of the tablet on the basis of the mineral complex and a calcium glycerophosphate. The analysis of the experimental data and their comparison with the spectra of the mixed saliva demonstrated that in the IR spectra of the preventive agents, one can see the groups of vibrations identical to those observed in the spectra of mixed saliva. It is due to the presence of similar organic–mineral groups and complexes in the composition of these preventive agents. Thus, application of the chosen preventive agents must be revealed in the changes of an organic–mineral balance of the mixed saliva and it can be observed in the IR spectra of the investigated samples.

Based on the proposed assumptions and using IR spectroscopy data, the above-named changes in the organic–mineral balance can be explored by the calculations and analysis of mineral–organic and carbon–phosphate ratios between the mineral and organic components in the dry residue of the mixed saliva. To calculate the first ratio it is sufficient to consider the ratio of the integral area in the IR spectra (spectral regions at 900–1200 cm^{-1} and 730–770 cm^{-1}) to the integral area of vibration band 1615–1775 cm^{-1}, related to Amid I. The carbon–phosphate ratio can be calculated from the ratio of intensity of vibration bands for C=O and CH_2/CH_3 bonds, localised in the range of 1430–1400 cm^{-1}, to the intensity of phosphate bands in the IR spectrum (900–1200 cm^{-1} and 730–770 cm^{-1}). Calculations of these ratios were performed with the use of the software OPUS (Bruker).

The results of the calculations are presented in Table 2. Since the chosen technique of analysis (IR spectroscopy) allows one to make only semi-quantitative conclusions on the sample composition, in Table 2 there are only relative changes in mineral–organic and carbon–phosphate ratios (relative to the values of these ratios calculated for the reference samples at the initial stage of the investigations). It should be noted that the above-named ratios are averaged over the groups of participants taking part in the investigations for different stages of the experiment.

Discussion

Taking into account the regulation of the experimental performance in the second stage of the investigation and also analysing the obtained data (see Table 2), it is possible to state that an oral rinsing after tooth brushing of the oral cavity with the use of the toothpaste with the multi-mineral complex involving calcium glycerophosphate completely nullifies its positive effect directed at changing the organic–mineral balance of the mixed saliva. Oral rinsing 30 min after the application of the toothpaste for oral cavity hygiene procedures showed the same effect. In both cases, one can observe a negative relative change of the organic-mineral ratio as compared with the reference sample, thus meaning washing-out of the mineral groups and complexes remaining in the oral cavity after the application of the toothpaste.

Analysing results of the changes of mineral–organic and carbon–phosphate ratios after the patients have taken the tablets containing glycerophosphate, one can conclude that in the first stage of the experiment (after taking the tablets for three days) an increase in the mineral–organic ratio of 12% was observed as compared with the reference sample of the mixed saliva.

As for the carbon–phosphate ratio, it was reduced by almost two times on average after the application of the tablets containing calcium glycerophosphate.

All of the above-described facts mean that the organic–mineral balance in the mixed saliva following the use of tablets of a multimineral complex with calcium glycerophosphate shifted to more mineral groups and complexes. It should be emphasised that, to the best of our knowledge, the content of endogenous biologically available phosphate groups and complexes in the oral fluid is 12% higher than that in the oral fluid prior to the use of prevention measures (in the sample). It should be noted that it is in this case that we observe a long-term effect of mineral groups on the enamel surface obtained using endogenous methods, which we believe to be beneficial for remineralising therapy and to contribute to caries resistance of the dental enamel.

From the results of our work, we can state that certain correlations between the use of endogenous and exogenous methods of caries prevention and the changes in mineral–organic and carbon–phosphate ratios within the dry residues of mixed saliva were found for the groups of patients participating in the experiment. These changes make it possible to assume that exogenous ways of prevention provide only a short-term effect of maintaining the balance between the agents in mixed saliva required for the remineralisation process. At the same time, endogenous methods result in the long-term presence of the mineral groups and complexes necessary for providing the corresponding conditions, i.e., they better saturate the mixed saliva with the mineral ions and complexes necessary for remineralisation of the dental enamel.

Statistical analysis

Statistical analysis of the changes in mineral–organic and carbon–phosphate ratios in the dry residues of mixed saliva for the groups of patients was performed with the use of the professional software for the statistical analysis SPSS v. 19 for Windows, SPSS Inc., Chicago, Illinois, USA).

Conclusion

Now let us try to answer the main question asked at the beginning of the work. Does dentifrice provide the necessary saturation of ions in mixed saliva to favour remineralisation?

Based on the results of the two experiments generalised for the groups of patients participating in the investigations, we can state that the use of only toothpaste with the multi-mineral complex involving calcium glycerophosphate for the prevention measures concerned with the incorporation of hard dental tissues is completely insufficient, since this way of prevention has only a very short-term effect of saturating the mixed saliva with mineral ions and complexes. Moreover, a thorough oral rinsing after prophylaxis with the use of a toothpaste completely removes the effect of saturation of the mixed saliva with mineral groups and complexes.

On the contrary, the use of tablets with a mineral complex and calcium glycerophosphate together with the exogenous prevention measures results in increased content of the mineral groups and complexes (phosphates) in the mixed saliva by on average 10%. From our results, the efficiency of this method of prevention seems to be higher than that of exogenous prevention owing to the possible long-term effect of optimal concentrations of calcium, phosphate and fluorides on the enamel surface. It should be added that it is in this case when the mixed saliva contains endogenous and biologically available phosphates derivatives, which can enhance active remineralisation of the dental enamel.

Finally, it should be noted that the conditions established for increased content of the mineral groups and complexes (phosphates) in the mixed saliva, i.e., for the activation of remineralisation processes, are prerequisites for their advanced concentrations in the biofilm on the surface of enamel. In this case, conditions for the active saturation of biofilm at the optimal concentrations as well as the activation of the following stages for remineralisation are to be further studied.

Acknowledgements

This work was supported by the grant of Russian Science Foundation, grant number 16-15-00003.

The part of this research was undertaken with The Infrared Microspectroscopy (IRM) beamline at the Australian Synchrotron, Victoria, Australia.

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