Advances in Medical Sciences 63 (2018) 185–191

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Review article

Human saliva as a diagnostic material

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ARTICLE INFO

Article history:
Received 10 July 2017
Accepted 9 November 2017
Available online 14 November 2017

Key words:
Saliva
Diagnostics
Glycomarkers
Mucins
Lysosomal exoglycosidases

ABSTRACT

Today blood biochemical laboratory tests are essential elements to the diagnosis and monitoring of the treatment of diseases. However, many researchers have suggested saliva as an preferable diagnostic material. The collection of saliva is simple, painless, cheap and safe, both for patients and medical staff. An additional advantage of saliva is the fact that it may be retrieved several times a day, which makes repeat analysis much easier. Furthermore, saliva has very high durability. Although 94–99% of salivary content is water, saliva also contains numerous cellular elements and many organic and inorganic substances, including most biological markers present in the blood and urine that may be used in the early detection and monitoring of many dental and general diseases.

Contents

1. Introduction

Biochemical laboratory tests are an essential part of human disease diagnosis and monitoring. For biochemical analysis various biological fluids or tissues are collected, however, blood is still the most commonly used diagnostic material. Unfortunately, blood collection is an invasive procedure that may involve some risk to the health of medical staff and patients (e.g. HIV, HBV) as well as a very large discomfort for many groups of patients. Therefore, many researchers have recommended saliva as the ideal non-invasive diagnostic material. Human saliva may be used in the early diagnosis and monitoring of many systemic diseases (e.g. cancer, infectious or cardiovascular disorders) [1], in the pharmacokinetic studies, in therapeutic drug monitoring [2,3]. Using saliva as a diagnostic material is possible, because a number of major inorganic and organic substances (e.g. proteins, carbohydrates

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https://doi.org/10.1016/j.advms.2017.11.002
1896-1126/

and lipids) as well as drugs and their metabolites are secreted into saliva. In addition, salivary collection is painless, easy, inexpensive, and completely safe for patients and health professionals [4–7]. Therefore, the analysis of the concentrations of various salivary components is becoming increasingly important in the laboratory medicine for diagnosis and monitoring of many oral [2,5] and systemic disorders [8–11].

2. Saliva

Saliva – a fluid excreted by the large and small salivary glands, is one of the most important factors affecting the homeostasis of the oral cavity. Salivary composition and secretion depends on the gland from which saliva is secreted, as well as a patient's age, gender and type of stimulating factor [11–13]. Human saliva is composed mainly of water (94–99%), however an important fraction of saliva is made up of proteins (especially glycoproteins) and lipids. Saliva is also rich in carbohydrates, salts and contains non-protein nitrogen (urea, uric acid, amino acids and creatinine) [14]. Besides the excretions of salivary glands, saliva includes gingival fluid, serum components, bacteria and bacterial metabolites, exfoliated epithelial cells and leukocytes. Adults secrete about 0.5–1 liters of saliva daily of which 80% is due to food intake. The secretion of saliva is controlled by the autonomic nervous system (Fig. 1). Quantitative and qualitative changes of saliva are also caused by a variety of oral and systemic diseases, for example: Alzheimer's disease [15], diabetes [13], cystic fibrosis [16,17] and oncological diseases (especially head and neck tumors) [6,8].

A major role of saliva is to create a protective environment for teeth and oral mucosa against a variety of harmful mechanical, biological and chemical stimuli. In addition, saliva takes part in the initial phase of food digestion and participates in the perception of taste. Saliva exhibits antibacterial, antifungal and antiviral properties that are conditioned by the presence of salivary immunoglobulins as well as innate immunity proteins, such as lactoferrin [18] and lysozyme [19]. Waszkiewicz et al. [18,19] reported a decrease in salivary lactoferrin [18] and lysozyme [19] output in chronically intoxicated alcohol-dependent patients, in comparison to social drinkers. Decrease in salivary lactoferrin and lysozyme output in chronically intoxicated alcohol-dependent persons, reflects the inhibition of synthesis and an increase in lactoferrin and lysozyme catabolism, caused by harmful action of ethyl alcohol and its toxic metabolites e.g. acetic aldehyde. Decrease in output of salivary immunoproteins induces deterioration of the paradontium of chronically intoxicated alcohol-dependent persons, in comparison to social drinkers [18,19]. Protection of the paradontium from harmful agents, next to immunoglobulins include enzymatic (e.g. peroxidase, catalase) and non-enzymatic antioxidant activity created by e.g. uric acid, polyphenols, ascorbic acid, reduced glutathione or albumin, protecting oral cavity against free radicals and other environmentally derived oxidative stress-induced agents [20]. Reduction of antioxidant activity can lead to the onset of inflammation in the oral cavity [21–25].

3. Salivary glands

Saliva is produced and secreted by the large salivary (parotid, submandibular and sublingual) as well as 800–1000 minor salivary glands located throughout the oral mucosa (Fig. 1) [4,26]. Cells of the salivary glands produce mucinous or serous human saliva. We may distinguish purely serous salivary glands (e.g. parotid or von Ebner's glands), purely mucous salivary glands (i.e. the glands located on the palate and base of tongue) and mixed (tubulo-alveolar) salivary glands. The mixed salivary glands include: the submandibular, sublingual, labial, buccal and molar salivary glands [27,28].

Large and medium size salivary glands have a lobular structure. The majority of human salivary glands are constructed with segments generating secretion (one layer of cuboid secreting cells wrapped with dense net of blood vessels) and tubes secreting saliva into the oral cavity. Individual salivary glands differ mainly in the structure of secretory segments and the type of produced saliva. Small salivary glands may be histologically distinguished from large and medium size salivary glands, as they are devoid of lobular structure and connective tissue capsule [27–30].

3.1. The parotid gland

The parotid gland is the largest (15–30 g), usually single (occasionally, there is an additional parotid gland) salivary alveolar gland, with a typical serous nature, located on the lateral side of the oral cavity [29] (Fig. 1). The parotid gland is found located in the vicinity of the submandibular salivary gland, separated by the bands of connective tissue. The parotid gland is surrounded by a capsule of connective tissue, divided by numerous septa connected with capsule and variable amounts of fat tissue. The salivary duct located at the frontal edge of the parotid gland perforates buccal

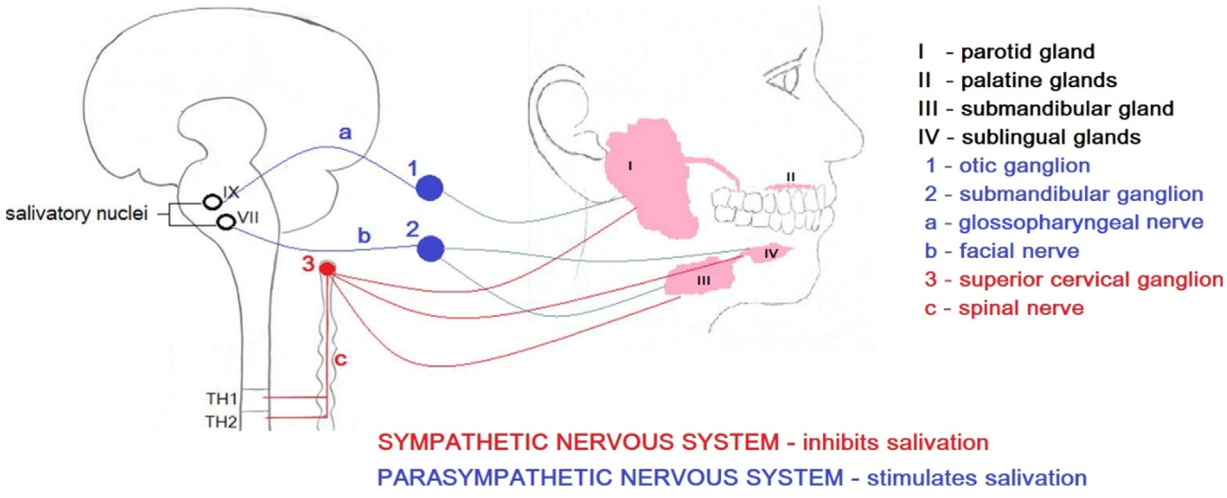

Fig. 1. Salivary glands and their vegetative innervation.

muscle and leads to the vestibule of the oral cavity at the region of the second cheek tooth. The parotid gland is parasympathetically innervated by the glossopharyngeal and sympathetically by spinal nerve (Fig. 1) [21,29].

3.2. The submandibular tubular-alveolar gland

The submandibular tubular-alveolar gland is located in the bottom corner of the submandibular triangle and is much smaller (7–16 g) than the parotid gland (Fig. 1). The submandibular gland has a harder consistency than the parotid gland and a mixed (serous-mucous) secretion. The submandibular salivary gland consists of pure alveolar, alveolar with serous tubules of a crescent shape and mixed salivary glands (however, majority of the submandibular gland releases serous saliva). The submandibular gland is parasympathetically innervated by the facial nerve and sympathetically by spinal nerve (Fig. 1) [30].

Sublingual glands are the smallest salivary glands (3–5 g) (Fig. 1), located at the bottom of the oral cavity, covered by mucous membrane. Sublingual glands consist of 5–20 small, separate glands. Sublingual glands represent mixed salivary glands, with structure similar to the submandibular gland, they secrete mostly mucus [29,30].

4. Methods of the salivary collection

The simplest method of collection of human saliva is sampling passively naturally flowing saliva from mouth to glass or plastic containers. Saliva may be also collected by aspiration or drainage of the outlet of the output ducts of the particular large salivary gland. The absorption method of the human salivary collection depends on mastication of the absorptive, usually cotton swabs. The absorption method is not recommended, as it carries a risk of permanent absorption of some substances by cotton swabs [4,31]. Although, methods of salivary collection may have significant influence on the precision and determination of biomarkers, up to now, there is no established uniform criteria for the collection of human saliva.

Human saliva is predominantly collected by spitting, where the patient spits out saliva from the bottom of the oral cavity into a glass or plastic calibrated container [32]. During the collection of saliva, a calibrated container (e.g. centrifugal tube) should be immersed in ice-bath to prevent degradation of biologically important salivary analytes [6]. To minimalise the influence of circadian rhythms on the results of salivary biochemical determinations, saliva should be collected between 8 and 10 AM [7]. Patients should not eat or drink any beverages (with exception of the pure water) as well as not perform any hygienic procedures inside the oral cavity (e.g. teeth brushing) for at least two hours before salivary collection. Because of the influence of many drugs on salivary secretion, patients should not take drugs at least 8 h before salivary collection [12]. Saliva should be collected in a separate room, from a sitting, relaxed patient with their head slightly bent down, with minimal movement of the face and lips, after 5 min of adaptation to the environment [32,33]. Salivary excretion may be stimulated by dropping citric acid solution at the edge of tongue [32,33].

5. Biologically important salivary components

Saliva derived from parotid glands (26% of total salivary volume) contains amylase and other digestive enzymes. Saliva derived from submandibulary glands (69% of total salivary volume) contains glycoproteins [4,26]. The most important constituents of saliva are glycoproteins [34] including mucins [35] and non-mucinous proteins [36]. Macromolecular glycoproteins (mucins) make up about 26% of salivary proteins. Salivary mucins play a significant role in the homeostasis of the oral cavity by creating an acquired membrane, covering the soft and hard tissues lining the oral cavity. Mucinous saliva lubricates the oral cavity walls and food during mastication. It forms mouthfuls and plays an important role in word articulation during speaking [6,8]. Human saliva contains: oligomeric mucin (MG1) with molecular mass above 1 MDa and monomeric mucin (MG2) with molecular mass of 200–250 kDa. Monomers of MG1 and MG2 contain heavily O-glycosylated tandem repeats located at the central domain of the molecule. MG1 monomers are oligomerised by disulfide bonds located at sparsely glycosylated N- and C- ends of their polypeptide chains (Fig. 2). Human MG1 are synthesized by the mucous cells and MG2 by the serous cells of the salivary glands [35]. Oligomeric structure of MG1 creates salivary viscosity and faster deposition of dental plaque impeding teeth cleaning. MG1 dominates in the saliva of patients with intensive caries. Monomeric MG2 predominates in caries resistant persons. MG2 responsible for bacterial agglutination, has antiviral and antifungal properties [37–41].

Oligosaccharide chains of salivary mucins are built by individual sugar molecules linked to each other O-glycosidically. The majority of oligosaccharide chains of human salivary mucins are O-glycosidically attached to polypeptide core [35] (Fig. 2). Glycoconjugates and their structures are important in physiology and pathology of oral cavity, however their applications in diagnostics of human diseases are minute. It is worth of note that glycosylation changes of salivary glycans may be used for monitoring alcoholic abstinence [22]. O-glycosidic linkages of glycoconjugates (glycoproteins, proteoglycan and glycolipids) are hydrolysed by lysosomal exoglycosidases [43]. Exoglycosidases hydrolyse: β-anomerically linked N-acetyl-D-*hexo*samines (*N*-acetyl-β-hexosaminidase- HEX, NAG), β-anomerically linked galactose (β-galactosidase (GAL), α- or β-anomerically linked mannoses, (α- or β mannosidase –MAN), α- anomerically linked fucose (α-fucosidase – FUC) and β-anomerically linked glucuronic acid in glycosaminoglycans (β-glucuronidase – GLU) [44–48]. Reduced activity of lysosomal exoglycosidases in saliva of healthy persons increases in dysfunction of salivary glands during many systemic diseases such as inflammation and cancer [49,50].

Most active of the lysosomal exoglycosidases, is N-acetyl-β-hexosaminidase (HEX, NAG) that hydrolyses N-acetylglucosamine and N-acetylgalactosamine from non-reducing end of glycoconjugates (glycoproteins, glycolipids and glycosaminoglycans) oligosaccharide chains [51]. HEX hydrolyses β-glycosidic linkages of hexosamines in natural and artificial substrates (e.g. derivatives of N-acetylglucosamine and 4-nitrophenol or 4-methyl-umbelliferone). Derivatives of 4-nitrophenole of hexosamines and other sugars are suitable for colorimetric and 4-methylumbelliferone derivatives are suitable for fluorescent determination of HEX and other exoglycosidases [52,53]. HEX may be electrophoretically separated on isoenzymes: B, I1, I2, P, A, S, C. HEX isoenzymes A and S are thermolabile; izoenzymes B and P are thermostabile [54]. In tissues HEX isoenzymes A and B have the highest activity, however their relative activities are different depending on the tissue. Thermolabile HEX A is inactivated by incubation at 50°C in pH 5.0 for 3 h. Differences in heat stability were used for colorimetric determination of HEX A and HEX B [55,56]. Remaining lysosomal exoglycosidaes (FUC, MAN, GAL, GLU) demonstrate lower specific activity than HEX, both in tisssues and body fluids.

HEX is a sensitive marker for stomatological [40,57] and general diseases [58–60]. A significant increase in the specific activity of HEX and its isoenzyme A was reported in the saliva of smoking and non-smoking alcoholics, probably as a result of the oral cavity inflammatory states [22,61,62]. Smoking alcoholics had higher HEX A specific activity in comparison to social drinkers [58,63].

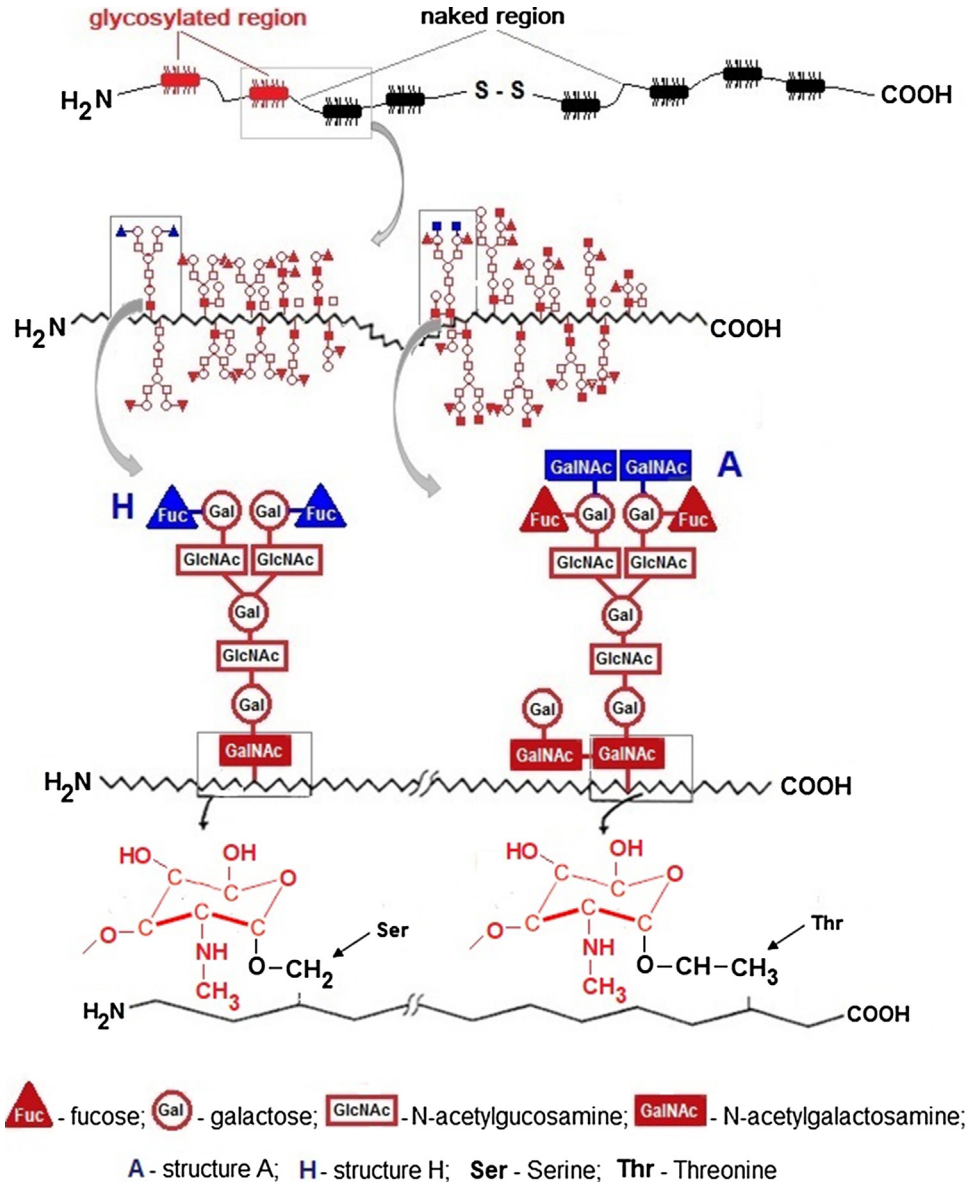

Fig. 2. Scheme of human mucin [42].

Alcohol abuse also increases salivary specific activities of the remaining exoglycosidases (FUC, GAL, GLU and MAN) [59,64].

Persons with alcohol abuse accumulate acetaldehyde and ROS (Reactive Oxygen Species) in oral cavity tissues [65]. Increase of ROS damages DNA, disturbs antioxidative processes that cause oxydative stress. A recognized marker of oxidative stress DNA damage is 8-hydroxydeoxyguanosine (8-OHdG) – a recommended salivary biomarker of paradontal diseases [66,67]. Alcohol abuse leads to non-inflammatory salivary gland edema (sialosis) with accompanying lipid accumulation in salivary gland cells and the decrease in potassium and total protein concentration. As a result of sialosis, degeneration of cells and atrophy of salivary glands was observed with the decrease in saliva production and the decrease in defense ability of oral cavity tissues. As a consequence of sialosis there is an increase in the number of teeth with caries [68,69]. Alcohol related degradation of oral epithelial cells is accompanied by the release of lysosomal enzymes to saliva. Increase of concentration of eptihelial cells lysosomal exoglycosidases disturb the balance between degradation and synthesis of glycoconjugates in tissues [70], aggravating pathology of the oral cavity. Enzymes released into saliva may be a useful source of biomarkers reflecting the status of salivary glands, not only in caries, but also in the course of arthritis [60] and neoplasma [71]. Pleomorphic adenoma (PA) is the most frequently occurring neoplasm of human salivary glands. PA is a benign neoplasm with tendency to malignancy and HEX and its isoenzymes of resected PA tissue may be valuable markers to evaluation tendency of PA to malignancy [72].

Increase of salivary exoglycosidases specific activity was observed in insulin dependent (Type 1) [73] and independent (Type 2) [74] diabetes mellitus, and gestational diabetes [75]. Diabetic increase in specific activity of the salivary exoglycosidases is a marker of increased degradation of glycoconjugates (glycoproteins, glycolipids and proteoglycans) of salivary glands and tissues of the oral cavity [73–75] that may suggest application of special stomatological prophylactic procedures to diabetic patients. It was found that smoking diabetics had higher specific exoglycosidase activity than non-smoking diabetics, as smoking

detriment saliva that additionally aggravated oral complications of diabetes [76].

Chronic inflammation of soft tissues of oral cavity is accompanied by accumulation of inflammatory cells (neutrophils, lymphocytes and macrophages) that take part in degradation of oral soft tissues. Inflammatory and dystrophic changes in endothelial cells of the oral cavity increase specific activity of lysosomal proteases and exoglycosidases. Increase of glycoconjugates catabolism reflected by increase in specific activity of salivary exoglycosidases, may explain the susceptibility of oral mucosal cells to bacterial, fungal and viral infections, which were found in patients with HIV [77,78].

6. Saliva as a diagnostic material

Saliva contains many disease markers which reflect the state of health of not only the salivary glands and oral cavity, but also the whole body. Saliva contains numerous cells (exfoliated epithelial cells, leucocytes, bacteries), many organic (peptides, proteins and among them enzymes) as well as inorganic substances (cations of calcium, magnesium, sodium, potassium; phosphate and carbonate anions). Saliva contains the majority of blood disease markers (antibodies, interleukins, neoplasma markers) that may be applied in the detection of early pathological changes in humans (e.g viral infections, autoimmunological diseases, cancers) [1,6,8,11,79]. Saliva may significantly facilitate biochemical and toxicological diagnostics in children and adults along with easy, non-invasive and convenient sample collection. Saliva constitutes an excellent alternative for blood, especially in patients with blood clotting disorders [80]. Saliva can be considered to be the best research material for scientific investigations on humans from the ethical point of view. Saliva is a very convenient diagnostic biological fluid, because it does not coagulate, is stable for diagnostic purpose for 24 h in room temperature and for a week at 4 °C [2]. Human saliva may be collected more often than other biological fluids, several times a day, facilitating repeat analysis during monitoring therapy. Saliva is a better diagnostic screening material than blood, as many people suffer from anxiety during blood collection. The fact that collection of saliva is better tolerated than collection of blood may result in less resistance to visit a diagnostic biochemical laboratory, earlier diagnostics and a decrease in expenditure of the budget for health.

A review of literature suggests that human saliva has been successfully employed in diagnostic of many systemic diseases such as:

- cancers e.g. ovaries [81], lung [82], breast [83], pancreas [84]
- autoimmunological diseases as: Sjörgen's syndrome, celiac disease [85,86], Hashimoto thyroiditis [87].
- infectious diseases: HIV and viral hepatitis [11], malaria [88], denga virus [80],
- endocrinological diseasses: diabetes 1 and 2 types as well as Cushing's syndrome [89,90],
- diseases of gastrointgestinal tract, e.g. gastroesophageal reflux [91].

Currently saliva is used in toxicological diagnostics e.g. detection of drug dependence and alcohol abuse [18,59,61,64,70], neurology, psychiatry [92–94] and forensic medicine (DNA) [95]. The concentrations of drugs in saliva correlate with the concentrations of drug in the blood [96–100]. Saliva can also be used as a diagnostic material in the analysis of the environmental exposure to fluorides [101].

In conclusion, it is felt that saliva is recommended as an excellent material for biochemical, toxicological and immunological diagnostics of the oral cavity and systemic diseases.

Conflict of interests

The authors declare no conflict of interests.

Financial disclosure

The authors have no funding to disclose.

Acknowledgment

We are indebted to Dr. Tony Merry of Oxfordshire for improving the style of the manuscript.

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