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Original article

Evaluation of taste related genes (CD 36, SHH, PLCß2) expression in saliva

Elif Esra Ozturk[1,2], Huseyin Karacan[3], Derya Dikmen[1]

[1]Hacettepe University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Ankara, Turkey; [2]Kilis 7 Aralik University Yusuf Serefoglu Faculty of Health Science, Department of Nutrition and Dietetics, Kilis, Turkey; [3]Diagen Laboratories Ltd, Ankara, Turkey.

Abstract. Due to its non-invasiveness accessibility and high availability in diagnostic and prognostic evaluation, the use of saliva has gradually gained popularity in recent years. This study's objective was to examine the expression level of taste-related genes in saliva (SHH, CD 36, PLCß2). The study was carried out in two stages. In the first stage, both lemon-juice stimulated and unstimulated saliva was gathered from 10 participants (6 males and 4 females) to examine the effect of stimulation with citric acid on expression level. Forty-six subjects (22 males, 24 females) participated in the second stage, and unstimulated saliva was collected due to the quality and yield of the mRNA obtained to examine the expression level of taste-related genes (SHH, CD 36, PLCß2). There was no difference between SHH expression levels in saliva stimulated with lemon juice and unstimulated saliva. The results showed that, SHH, CD 36, PLCß2 expression was higher in unstimulated saliva ($p>0.05$). The study demonstrated that unstimulated saliva is more suitable for determining taste-related gene expression levels.

Keywords: unstimulated saliva, lemon-juice stimulated saliva, gene expression, taste.

Introduction

Saliva is a valuable oral fluid that has increasingly come into greater prominence in recent years (1). It is more preferred than other biological fluids for research purposes since it is cheaply available and lacks invasive procedures – providing an accessible oral cavity ecosystem. It has also become a useful systemic sampling measure (1, 2).

Saliva is a mixture comprising the major (submandibular, sublingual and parotid) secretions and minor salivary glands and the crevicular fluid, bacteria, cells and food debris, and is constituted of approximately 99% water (2, 3). Saliva composition and flow vary depending on endogenous factors such as circadian rhythms, age, sex and disease-related conditions or exogenous variables, including diet and pharmacological agents (3, 4).

Recent research on saliva has demonstrated its immense potential as a diagnostic fluid for numerous diseases, including COVID-19 (2, 5-9). Furthermore, saliva has a significant role in the tasting process. Proper mastication helps release taste stimuli from the food matrix to the saliva phase and their transfer to taste receptors, during which several saliva constituents chemically interact with taste substances (3). In addition to facilitating the perception of flavor chemicals, saliva also plays a role in protecting taste receptors (10). The composition and properties of saliva greatly vary among individuals and throughout life. A more profound comprehension of the differences in salivary composition and properties may explain variations in taste perception. However, the precise mechanisms of how saliva affects and alters taste perception are a complicated and demanding research subject (3). Nevertheless, some gene expressions may play an

important role in taste perception, like Phospholipase C beta 2 (PLCß2) as a taste tissue marker, CD36 as a taste receptor gene, and SHH acting as a taste bud growth factor.

Due to the significance of saliva in tasting perception, this study aimed to identify the type of saliva more suitable in taste research and determine the expression levels of genes associated with taste perception (SHH, PLCß2 and CD 36).

Methods

Participants:

This study examined taste-related gene expression levels in saliva and was carried out in two stages. In the first stage, both stimulated and unstimulated saliva samples were gathered from ten participants (six males, four females, mean age 26.52±5.03) and the effect of lemon juice (containing 2-4% citric acid) on the expression level was investigated. The second stage involved the determination of SHH, CD 36, PLCß2 expression levels in the most suitable saliva for the analyze which investigated in the first stage. In the second stage, the study was conducted with 19-44-year-old forty-six subjects (22 males, 24 females, mean age 28.24±7.26) non-smokers free from any metabolic or chronic diseases. Female participants only with regular menstrual cycles that were not pregnant or breastfeeding were included in the study. This study's ethical approval was provided by the Non-Intervatinal Clinical Studies Ethics Board of Hacettepe University (GO 19/1060). Informed consent was obtained from each participant before the collection of the saliva.

Saliva Samples Collection

The participants were instructed not to eat and avoid oral hygiene activities for at least an hour before collecting samples (saliva). They were only allowed to drink water. All saliva collection sessions were carried out, in the same clinical room and at the same time, between eight to ten in the morning to minimize diurnal variations associated with saliva sampling. In order to prevent contamination, 5 mL saliva samples were gathered in 50 mL DNase-RNase Free Falcon tubes (11). The tubes were kept on ice during collection and were transported in an ice battery maintained at -80°C to isolate RNA until analysis (12). In the first stage, both stimulated, and unstimulated saliva was gathered from ten participants. Stimulated saliva was collected from the same participants half to one hour after unstimulated saliva was collected.

Unstimulated saliva collection

The participants were rested and calmed to adapt to the environment before saliva collection. They were asked to rinse their mouth with distilled deionized water for debris removal and moisturization ten minutes before commencing collection activities (13). Saliva was gathered with the draining method (14) in a position postulated to be ideal - sitting erect, head slightly leaning forward, and eyes open. The participants were requested not to talk during the procedure (13, 14). The participants sat gently, with their heads slightly tilted forward and mouths open to facilitate passive dripping of saliva from the lower lip into sterile graduated tubes (14).

Stimulated saliva collection

Freshly squeezed five mL lemon juice concentrate (contains 2 - 4% citric acid) was used as a stimulant (15). Participants were instructed to swill freshly prepared lemon concentrate around in their mouths for 15 seconds without swallowing. After that, their saliva was collected for 5 minutes in DNase-RNase Free Falcon tubes (16, 17).

RNA extraction from unstimulated and stimulated saliva

After incubation on ice for 10 minutes, saliva samples (500µl) were centrifuged for 5 min at 12000g, and the cell-free clear supernatant was pipetted to a new Eppendorf tube while the separated pellet was discarded.

To decide on the quality and the quantity for total RNA extraction, RNA was extracted with both column-type (Vivantis Total RNA Extraction Kit, Kurabo Tissue RNA Extraction Kit) and Trizol-based

(GeneAll RiboEx RNA Extraction Solution) methods according to the manufacturer's instructions. GeneAll RiboEx RNA Extraction Solution's relevant protocol was carried out, and RNA pellets were washed twice after adding 1 mL 75% ethanol to remove contaminants. The extracted RNA samples were stored at -80°C. Before measuring the total RNA amount, Biolabs M DNase I (USA) enzyme and buffer were used to prevent DNA contamination. To DNase I inactivation, 1 **µl** Vivantis 0.5 mM EDTA solution (Malaysia) was used, and RNA was dissolved in 50 µl RNase-free water. RNA samples extracted from saliva samples were analyzed in terms of RNA quantity using Colibri Microvolume Spectrometer (Titertek-Berthold) with the acceptable quality determined by absorbance ratio measurement at 260nm/280nm and the relation of 1.5 to 2 was considered an indicator of suitable purity degree. All steps were performed under RNase-free conditions.

Target cDNA Preparation

Complementary DNA (cDNA) synthesis from total RNA was performed with ABM OneScript® Plus cDNA Synthesis Kit (Canada). In addition to gene-specific primers, random primers and Oligo (dT) was used. Complementary DNA was synthesized from the total RNA using OneScript® Plus RTase by matching random hexamer and Oligo (dT) to bind to mRNA. The cDNA obtained was stored at -20°C.

Quantitative Gene Expression Analysis by qPCR

RT–qPCR was completed on an Applied Biosystems 7500 Fast Real-Time PCR system using BrightGreen 2X qPCR Master-Mix. Amplifications were conducted with a total reaction volume of 20**µl containing cDNA (1000 ng/reac**tion), mRNA-specific primers (5µl), BrightGreen qPCR MasterMix (10µl) and nuclease-free water. Following 10-minute enzyme activation / initial denaturation at 95°C, 45 cycles were carried out for 15 seconds at 95°C and 60 seconds at 57°C. Melting curve analyses of amplicons generated by Real-Time PCR (Applied Biosystems 7500 Fast Real-Time PCR) revealed that the test and, consequently, the PCR products were specific.

Statistical data analysis

The cycle threshold (Ct) was determined as the cycle number at which a sample's fluorescence reaches a defined threshold. Gene expression data were analyzed based on the $2^{-\Delta\Delta Ct}$ method (18). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included as a reference gene for internal controls in normalizing gene expression. All reactions were performed duplicated, so results were calculated based on the average of the experiments performed. Statistical analysis was performed using IBM SPSS statistical software version 22 (SPSS Inc., Chicago, IL, USA). The results were presented as means (X) with standard deviation (SD) or medians with range (min-max). A chi-square test was performed to investigate differences in the purity of RNA to different RNA extraction kits. The Friedman test was used to compare the difference between quantifying and purity (A260/280) of RNA in unstimulated saliva, and the different group was determined by applying a Wilcoxon signed-ranks test. The Wilcoxon signed-ranks test was used to compare quantify and purity of RNA and $2^{-\Delta\Delta Ct}$ values in stimulated and unstimulated saliva samples. A p-value of ≤ 0.05 was considered statistically significant.

Table 1. The primers used in this study.

Genes	5′- Forward primer sequence-3′	5′- Reverse primer sequence -3′
Human -SHH	CGAGTCCAAGGCACATAT	GTGAGGAAGTCGCTGTAG
Human PLCß2	AACTCCATCAATCCTGTCTG	CTTGTTGCCTTCCTCCAT
Human CD 36	CAGGTCAACCTATTGGTCAAGCC	GCCTTCTCATCACCAATGGTCC
Human GAPDH	AATCCCATCACCATCTTCCAG	AAATGAGCCCCAGCCTTC

Results

The median RNA quantity was significantly higher in GeneAll RiboEx RNA Extraction Solution (535.50 ng/μL, range 338.00-568.00 ng/ μL) than the others (Vivantis Total RNA Extraction Kit: 1.05 ng/μL, range 0.20-12.00 ng/μL; Kurabo Tissue RNA Extraction Kit: 5.10 ng/μL, range 0.80-45.30 ng/μL; p<0.01). The quantity of RNA in stimulated saliva was lower, indicating that acid stimulation will negatively affect RNA in saliva (p <0.05). When the purity of RNA (A260/A280 ratio) was compared amongst the three groups in stimulated saliva, purity of the RNA in GeneAll RiboEx RNA Extraction Solution was higher (p>0.05) than the other samples (Table 2.). Regarding the quality of RNA harvested in unstimulated saliva, 90.0% of the samples were observed to be optimum 1.5 to 2.0 (A260 / 280) in the GeneAll RiboEx RNA Extraction Solution; The Kurabo Tissue RNA Extraction Kit met 30% with this condition and the Vivantis Total RNA Extraction Kit 20.0% (p = 0.003).

Therefore, GeneAll RiboEx RNA Extraction Solution was selected as the preferred method for RNA extraction. When RNA quantify and purity in unstimulated and stimulated saliva samples were compared, the unstimulated saliva was higher than the stimulated samples(p<0.05). Also, 70.0% of the samples were observed 1.5 to 2.0 (A260 / 280) in terms of the quality of RNA harvested in stimulated saliva. There were no statistically significant differences between unstimulated and stimulated saliva (p=0.582).

The median SHH expression level in unstimulated saliva was 1.17, where the minimum value was 0.00, and the maximum value was 45.89. The median SHH expression level in lemon juice stimulated saliva was 0.77, ranging from 0.09 to 7.78 (Fig. 1). There was no difference between the SHH expression levels in stimulated and unstimulated saliva (p= 0.139). According to the first stage analysis results, as the amount of

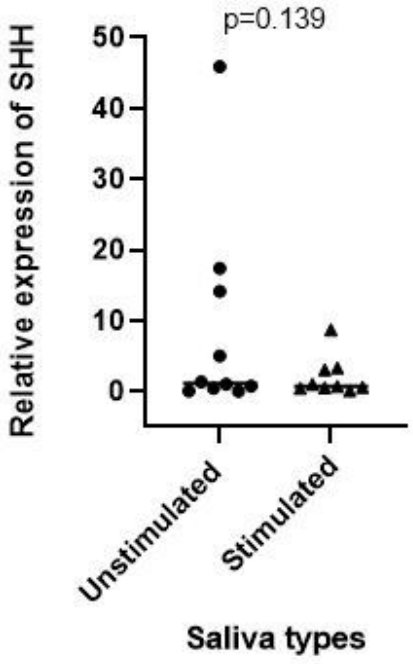

Figure 1. SHH Expression level according to the saliva type

Table 2. Comparison of RNA quantity and quality between unstimulated and stimulated saliva in preliminary analysis results

Preliminary analysis	Total RNA (ng/ul) Median (Range)	p	A 260/280 Median (Range)	p
Unstimulated saliva (n:10)				
Vivantis Total RNA Extraction Kit	1.05 (0.20-12.00)	<0.01	0.69 (0.25-3.84)	
Kurabo Tissue RNA Extraction Kit	5.10 (0.80-45.30)		0.92 (0.31-4.16)	
GeneAll RiboEx RNA Extraction Solution	535.50 (338.00-568.00)		1.79 (1.63-2.10)	0.082
Stimulated saliva (n:10)				
GeneAll RiboEx RNA Extraction Solution	75.50 (55.00-163.00)	0.005*	1.50 (1.25-1.79)	0.005*

* The p value of comparing stimulated and unstimulated saliva with GeneAll RiboEx RNA Extraction Solution.

Table 3. Total RNA quantity and SHH, PLCß2, CD 36 expression levels

Genes	Total RNA (ng/ul) Median (Range)	A 260/280 Median(Range)	Ct Mean ±SD	$2^{\wedge-(\Delta\Delta Ct)}$ Median (Range)
SHH (n:46)	597.00	1.85	29.84±2.03	1.13 (0.01-79.35)
PLCB2(n:46)	(262.00-1367.00)	(1.59-2.18)	31.16±2.54	1.32 (0.01-33.82)
CD 36 (n:40)			32.43±3.57	0.83 (0.006-61.84)
GAPDH(n:46)			28.99±2.48	-

RNA and purity levels were higher than the stimulated saliva, unstimulated saliva was collected in the second stage. Furthermore, real-time PCR linearity and melting curves in unstimulated saliva were also more linear and stable than stimulated saliva.

According to the first stage analysis results, SHH expression levels were also higher in unstimulated saliva. Moreover, the real-time PCR linearity and melting curve analyses were linear and more stable than the stimulated saliva.

In the second stage, quality of RNA harvested in unstimulated saliva, 84.78% of the samples were observed to be optimum 1.5 to 2.0 (A260 / 280) where the minimum value was 1.59 ng/μL, and the maximum value was 2.18. For the unstimulated saliva, the median of RNA was 597.00 ng/μL, ranging from 262.00 ng/μL to 1367.00 ng/μL. The median SHH expression level in unstimulated saliva was 1.13, where the minimum value was 0.01, and the maximum value was 79.35. The median of PLCB2 expression level was 1.32, with a range varying from 0.091 to 33.82, and the median CD 36 expression level was 0.83, ranging from 0.006 to 61.84 (Table 3.).

Conclusion

We compared the RNA extraction results and qRT-PCR from saliva samples according to the type of saliva samples. This study was conducted to contribute to taste-related gene expression studies in saliva. A detailed explanation of the methodology has been provided above. We believe that our process flow is useful and generally viable for generating reliable gene expression data from unstimulated saliva. The study results indicate the unstimulated saliva as a material that can be used in researching the expression of genes that feature in taste perception.

Conflict of interest: The authors declare that there are no conflicts of interest.

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Corresponding author:
Derya Dikmen
Hacettepe University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Ankara, Turkey.
Hacettepe University, Faculty of Health Sciences, Department of Nutrition and Dietetics,06100, Ankara, Turkey.
E-mail: ddikmen@hacettepe.edu.tr
Elif Esra Ozturk
Hacettepe University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Ankara, Turkey.
Kilis 7 Aralik University Yusuf Serefoglu Faculty of Health Science, Department of Nutrition and Dietetics, Kilis, Turkey.
Hacettepe University, Faculty of Health Sciences, Department of Nutrition and Dietetics,06100, Ankara, Turkey.
E-mail: elif.ozturk@hacettepe.edu.tr
Huseyin Karacan
Diagen laboratories Ltd, Ankara, Turkey
Diagen laboratories Ltd, 06110, Ankara, Turkey.
E-mail: h.karacan@diagen.com.tr