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RESEARCH ARTICLE

FIRST RESULTS OF AN IN VITRO STUDY ON THE EFFECTS OF GREEN TEA ON THE DEFENSIVE LAYER OF THE ORAL CAVITY

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ABSTRACT

Introduction, Objectives: The polymeric gel-forming mucins provide together with other proteins the structural framework of saliva and the mucus barriers that cover the mucosal surfaces of the oral cavity. The polyphenols content in green tea, which have many health benefits, are able however influence the barrier properties of these protective layers. The aim of this study it is that to verify in vitro such effects on two different samples of saliva

Materials Methods: In two male volunteers, asked to provide a saliva sample, obtained from the collection of the same, at various times of the day, they were determined the pH, density, total proteins and mucins. To these samples they have been assistant increasing quantities of extract of green tea, and then, after centrifugation, determined in the surnatant the concentrations of total proteins and total mucins. The results are analyzed with Fisher Exact Test and T –Test

Results: The precipitation of proteins and mucins in both samples after the addition of green tea extract are not statistically non-correlated data ($p \geq 0.05$), to the increase the amount of polyphenols compounds, but points out a tendency, by the Fisher Exact Test (FET). The results of this trend arising for the sample 1 by the maximum proteins precipitation value of 32,% and 57.6 %, for the mucins. Test 2 maximum precipitation for the proteins is 28.5%, and 50.0% for mucins. From the same results it is underlined instead that in both the champions the differences of concentration between the mucins and the proteins are statistically significant: the difference of the medium vale with T-TEST is ≥ 0 for both samples

Discussion: The two samples of saliva have different initial biochemical parameters values; this fact influence in the differences in the precipitation of the proteins in the two different champions, as it happens for the mucins, but, also the different behaviour between the same proteins and mucins to parity of green tea assistant extract. A value of more elevated, or more distant pH from the isoelettric point, it stretches the protein structures favouring the interaction of the same with the polyphenols, The mucins are in this case more receptive for the presence of polysaccharide terminals groups

Conclusions: These data demonstrate the possible effect of a dietary using of the green tea on the mucin network; given the diversity of human diet, many other dietary compounds may also affect the properties of mucus barriers at the level of the mucin network, but the polyphenols compounds are the most important in the alteration of mucus barrier properties in health and disease states, as demonstrated from this study and from various epidemiological evidence

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INTRODUCTION

The components that confer to the saliva of the barrier properties with viscoelastic characteristics are biopolymers or glycoprotein called mucins, The saliva which is a complex mixture of water, mucins, electrolytes, proteins, lipids, urea. And it is important for the phonation, mastication, lubrication, swallowing and to start the digestion, also forms a protective barrier for teeth and soft tissue (Varga, 2012). The oral film is about 100 nm thick, according to a recent study (Morzel *et al*., 2014), is composed of concentrated salivary proteins, including the salivary mucins (Gibbins *et al*., 2013) to form a film of saliva in addition to these layers, one layer of adsorbed protein is also observed to air-liquid interface of saliva (Proctor *et al*., 2005); MUC5B is the main gel-forming mucin, and is expressed by the cells acinar mucous submandibular, sublingual and minor salivary glands in the oral cavity. There is

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evidence that the different cells in these glands secrete different glycoforms of the same protein MUC5B Another mucin found specifically in saliva is much smaller, mucin not gelling, èMUC7, expressed and secreted by the mucous acinar cells of the salivary glands (Kouznetsova *et al*., 2010). MUC5B and you MUC7 are also concentrated in the oral mucosa film covering the soft tissue of the mouth (Gibbins and Carpenter, 2013). The mucinous polymers are assembled within secreting cells, packed into secretor granules; in intragranular conditions of low pH and high concentration of calcium such as to reduce and shield the negative charges of mucin oligosaccharides of lead to concentrate them and condense them into vesicles. After mucin secretion by exocytosis, after the shielding charge and the effect of a low pH and high content of calcium occurs the expansion of mucin structure. There is a model (Kesimer *et al*., 2010) proposed that provide that the expansion of mucins starts from, mucinous polymer, hydrophilic because of the oligosaccharide chains, and which are arranged in a random conformation due to entanglement and cross ties. Dietary components may affect the structure of the components of all the salivary proteins as in the case of world consumption of betel, which certainly involves the formation of oral cancers across large portions of Southeast Asia. Other compounds like. plant lectins, found in seeds, nuts, potatoes and beans, have the ability to bind to mucin and O-glycans and mucins cross-links in multilayer (Gibbins and Carpenter, 2013). Another important group of chemical compounds in foods and beverages of vegetal origin are polyphenols.: it is shown that the plant polyphenols present in enormous quantity as precisely in betel, (up to 35% by weight), in a wide consumption spices such as paprika, and finally in green tea, interact with the various salivary components. The structures of green tea polyphenols principally involved in this process are: epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG).

There are contrasting reports on the effect of EGCG on salivary mucins. Proteomic studies of human whole saliva mixed with EGCG suggest that mucins are not among those proteins precipitated by polyphenols (Lee *et al*., 2013). In sharp contrast, studies on poorly characterized commercially available mucins report their aggregation by polyphenols (Zhao *et al*., 2012). The use of native, polymeric mucins is needed to provide better insight into the interaction of native salivary mucins with EGCG. Is known that the green tea polyphenols EGCG causes the precipitation of various salivary proteins. The salivary proteins affected include PRPs, amylase, cystatins and histatins (Cala *et al*., 2012), and it is widely regarded that the precipitation of these proteins by EGCG are mechanisms by which EGCG contributes to the astringency (dry-puckering sensation) of green tea (Gibbins and Carpenter, 2013). Despite extensive research into salivary protein interactions with EGCG, the interactions of the salivary mucins, polymeric gel-forming MUC5B (the main structural component of saliva) and non-gel-forming MUC7, with EGCG had not been well studied, and the effects of EGCG on mucins were somewhat unclear. The aim of this study is to occur for the first time in what entails in vitro biochemical system complex of human saliva when you add to it, of green tea extracts in concentrations equal to those which are usually present in beverages consumed in a single time

MATHERIALS AND METHODS

Two male volunteers, all not smokers, were asked to provide a saliva sample, obtained from the collection of the same, at various times of the day. The volunteers were chosen from all non-smoking and previous anamnesis, showed no diseases that could not affect the value of salivary proteins concentration, such as for Sjögreen syndrome, nor that presented in physical examination oral disease such as gingivitis. The sampling was performed within two hours in the morning and also in the afternoon, without stimulators processes, taking care to provide saliva about an hour away from meals. The volunteers gave each a sample of whole saliva of approximately 60 ml, and for every test are employed 10 ml; of these samples was carried out biochemical analysis for total protein concentration (mg/dl), and after for pH value. The flow rate is measured from direct observation during the harvest in test tube graduate The concentration of total proteins was performed by photometric Biureto test while the pH and SGS/Poids was determinate by the use of sticks for urinalysis, type Uriscan Roche, reading the values with automatic analyzer URIYSIS 2400 ROCHE. Mucin concentration was determined using the Alcian blue method, briefly described here.The samples of saliva diluted (1:10) were incubated for 30 min in a 1% solution of Alcian blue in 50 mM sodium acetate buffer with 25 mM Cl2Mg, pH 5.8 under constant agitation at room temperature .After following the dissociation of mucin-dye complexes are s with the addition of a 1: 2 dilution of Aerosol OT (Sigma Chemical Co., St Louis, MO, USA) in distilled water, mixing short and ultrasound the extracted samples ethyl ether and after centrifugation the concentration of dye was determined spectrophotometrically at 605 nm in the aqueous layer.

The sample of green tea extract is provided by the company "MY PROTEIN "Ltd Manchester and named "MEGA GREEN TEA EXTRACT" with leaves of superior quality coming from a called plant Camellia Sinensis. This drawn out powerful person contains 98% of polyphenols in total and 45% of EGCG. The amounts of green tea extract are assistant to the different of saliva samples, shaken then centrifuged; in the surnatant liquid has been determined according to the methodic described the various parameters of interest. The results are statistically analyzed with Fisher Exact test, and T-test

RESULTS

In saliva samples obtained from the two volunteers we were determined the parameters whose values are shown in Tables 1 and 2. As can be seen, there are differences in all parameters considered with the exception of the amounts in absolute terms of the concentration of mucin fractions, are different from each other and the different pH value can affect the precipitation of the mucins and proteins. The adding green tea extract to the two different saliva samples immediately involves the formation of a flocculation process, (see Image 1), more or less evident depending on the amount of it. After centrifugation is observed the formation in all the samples of a sediment and a clear phase, in which were determined the parameters whose values are expressed in Tables 3,4

Table 1. Principal biochemical parameters in first healthy control

Sample	Sex male	Age Years 35	pH	Flow Rate ml/min	SG/POIDS	Total Protein mg/dL	Total Mucins mg/dL	Rate % Mucin proteins
1+2			7.4	1.0	1.005	300	30	
3+4			7.3	1.0	1.01	306	30.6	
5+6			7.4	1.05	1.005	298	29.8	
				AVERAGE				
Final			7.4	1	1.005	301	30.1	10.0

Table 2. Principal biochemical parameters in second healthy control

Sample 2	Sex male	Age Years 60	pH	Flow Rate ml/min	SG/POIDS	Total Protein mg/dL	Total Mucins mg/dL	Rate % Mucins Proteins
1+2			6.5	0.8	1.025	320.5	30.0	
3+4			6.3	0.77	1.025	322.5	30.0	
5+6			6.3	0.83	1.02	312.0	30.0	
				AVERAGE				
Final			6.4	0.8	1.023	318.5	30.0	9.5

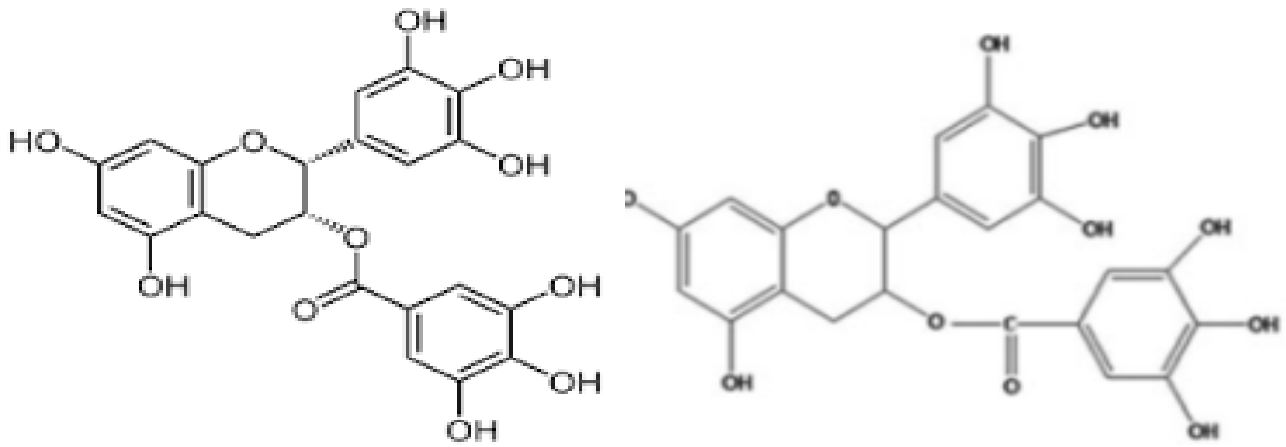
Table 3. Effect of the addition of green tea extract on saliva samples first in healthy control

Saliva ml	total proteins conc mg/dl	Protein mg/10cc	Total Mucin mg/dL	Mucins mg/10 cc	green tea mg	Protein Solution mg	Protein Precipit %	Mucin Solution mg	Mucins Precipitate %
10	300	30	38	3.8	50	24.6	18	2.6	30.4
10	300	30	38	3.8	100	22.8	24	2.5	37.0
10	307	30.7	38	3.8	150	22.1	28	2.1	45.6
10	305	30.5	38	3.8	200	21.1	30	1.7	54.3
10	295	29.5	38	3.8	250	20.1	32	1.6	57.6
10	300	30	38	3.8	300	20.2	32	1.6	57.6

]

Table 4. Effect of the addition of green tea extract on saliva samples second in healthy control

Saliva ml	Total Protein Conc mg/dL	Protein mg/10cc	Total Mucins mg/dL	Mucins mg/10 cc	Green Tea mg	Protein Solution mg	Protein Precipitate %	Mucin Solution mg	Mucins Precipitate %
10	315	31.5	30	3.0	50	27.7	12	2.1	28.5
10	326	32.6	30	3.0	100	26.7	18	2.0	34.5
10	330	33.0	30	3.0	150	25.5	22.7	1.8	40
10	315	31.5	30	3.0	200	23.0	26.9	1.7	44
10	310	31.0	30	3.0	250	22.2	28.1	1.5	49
10	315	31.5	30	3.0	300	22.5	28.5	1.5	50



Picture 1. EC structure EGCG structure

Image 1. Complex_Mucins_Proteins _Green Tea Extract Flocculation

Although the data in the amount of proteins and precipitated mucins are not statistically correlated, ($p \geq 0.05$ Fisher Exact Test), with the increase of green tea extract added to saliva, there is clearly a positive trend in the concentration of precipitate The both parametric tests in both The :; rho = 0.84 and rho = 0.67. Since data, in both cases, consist of two different measurements (percentage of precipitation of proteins and percentage precipitation of mucins) on same sample, applies a t test for paired samples. Built difference the sample between the data occurs if this has an average significantly different from zero, are normally distributed. The difference value in both test are positive with a one-tailed test application

For Table 1

Proteins Precipitation % : (18 ;26 ;28 ; 30 ; 32 ; 32)
Mucins Precipitation %: (30.4 ; 37.0 ; 45.6 ; 54.3 ; 57.6 , 57.6)
T-test (proteins precipitation/ mucins precipitation, paired = true)
Paired T-test for sample 1
Data proteins precipitation and mucins precipitation
t=-7,7795 ; df =5 ;p-value =0.0005618
alternative hypothesis :true difference in means is not equal to 0
95 percent confidence interval:
-26.27602 -13.22398
Sample estimates: mean of the difference =-19.75

For Table 2

Proteins Precipitation % :(12; 18; 22.7; 26.9; 28.1; 28.5)
Mucins Precipitation % : (28.5; 34.5; 40 ; 44 ; 49; 50)
Paired T-test for sample 2
Data proteins precipitation and mucins precipitation
t=-19.688; df =5; p-value =6.243e-^{06}
alternative hypothesis :true difference in means is not equal to 0
95 percent confidence interval:
-20.6894 -169106.
Sample estimates: mean of the difference =-18.3

DISCUSSION

The precipitation of proteins and mucins with tannin compounds is linked to the presence of polyphenol compounds and mainly from epigallocatechin-3-gallate (EGCG), present in high concentration in the extract of green tea, (in our sample ≥ 45%). In contrast, the green tea polyphenol epicatechin (EC), in many experiments did not cause aggregation of salivary mucins or porcine gastric mucins, suggesting That the galloyl ring of EGCG,, which is absent in the EC, (see picture 1), is important for its aggregation of mucins, and that EC has different mechanisms of astringency. The most abundant and astringent polyphenol in green tea is EGCG, which has been shown to interact with a number of salivary proteins (Narukawa *et al*., 2010). It is well documented that PRPs, cystatins, histatins and amylase interact with, and are precipitated by, EGCG (Cala *et al*., 2012; Stevenson and Hurst, 2007). The mechanism of EGCG-induced protein aggregation has been the subject of many studies, (Zanchi *et al*., 2008; Charlton *et al*., 2002a; Charlton *et al*., 2002b); the conclusions of which indicate that the stages involved are: the initial binding of EGCG to the protein; EGCG bridging of EGCG-bound protein molecules; and EGCG-induced protein aggregation. The initial binding interaction of EGCG and proteins has been explored by several research groups. and has been suggested that individual EGCG binding sites may be 1-2 amino acids long and that proline, arginine, phenylalanine and histidine have high affinity for EGCG (Charlton *et al*., 2002a; Charlton *et al*., 2002b). The binding of EGCG to proteins is said to be reversible and co-operative: there is evidence that hydrophobic interactions and hydrogen bonding are involved in the interaction (Maiti *et al*., 2004; Maiti *et al*., 2006), and hydrophobic stacking between EGCG rings and proline side chains has been reported, (Charlton *et al*., 2002a). The galloyl ring of EGCG is likely to be important for protein binding, In our two tests the percentage difference of precipitated proteins from the extract of green tea in different saliva samples, is statistically irrelevant., thug more precipitation of the same are still found in saliva at a higher pH: this fact beyond the unverified possible concentration difference of calcium ions in the two samples, probably due to the fact that the more the pH of the solution moves away from the isoelettric point, near to basic values (pH 7-7.5). The protein more they tend to hang their structure for the decrease of the charge of the acid groups (Menicagli *et al*., 2016; Menicagli *et al*., 2015). This hypothesis is confirmed by experimental data showing that until the pH value of eight, there is an increase of spinnbarkeit, if they are not modified for other causes of bicarbonate and calcium concentrations, (Vijay *et al*., 2015). This factor, together with the different structure of mucins compared to the proteins, rather than justifies the remarkable precipitation statistically significant difference, found by our tests, in both types of saliva. Since these residues are all found within the N- and C-terminal regions of human MUC5B, MUC7, it is likely that there are many binding sites for EGCG on these mucins, but coverage due to their glycosylation present in the central domain, leads to a more relaxed spatial conformation. The

evidence for this comes from the fact that has been experimentally verified that mucins have a greater radius of rotation in water as a function of time, compared to non-glycosylated proteins of similar length (Jentoft, 1990). In contrast to the protein domains of MUC5B, the oligosaccharide-rich regions of MUC5B were not aggregated by EGCG. Experimental observations have also shown that upon additions of EGCG, there was an INCREASE in muc5b viscosity at all MUC5B concentrations determinate, as well as MUC7. This increase in viscosity is related to strong structural variations of salivary mucins, which are aggregated by EGCG, even in low concentration, with a tendency of the mucin complex.-EGCG, to sediment, according to a set to the other salivary proteins, the proposed scheme can and 'very likely that he knows that training, at lower pH, ternary complex -mucin-EGCG proteins, which then precipitate

Conclusion

The principal function of the saliva in defense of the v oral cavity can only be guaranteed under certain conditions of the rheology that prevents the film salivary mucin, in the process of sliding and renewal, the phenomena of abrasion and laceration. Also note that if the saliva increases very its viscosity inhibits its same lubricating ability, preparing also in this case the oral cavity to various diseases. Ultimately mucins are essential in maintaining the lubricity, adhering strongly to the surface in layers, formed with the repulsive areas, by steric-electrostatic bounds. The concept of astringency, therefore indicating a phenomenon which results in the precipitation of all salivary protein with also mucins, and is induced primarily by polyphenolic compounds, In practice, the seizure of the proteins by the tannins is because the presence of amino acid groups and this involves the formation of an aggregate very stable and insoluble in water due to the formation of bonds between the OH groups present in tannins with oxygen ketoimidic bond (-CO-NH-) peptide of amino acids (Menicagli *et al*., 2014). The same mechanism underlying the precipitation of the salivary mucins This process becomes much more relevant to the presence in the core of proteic structure of the sugar portion of the fruit of glycosilation. That process it tends to assume the mucins a very expanse spatially structure in the middle watery saliva which facilitates interaction with polyphenols .The end result is as stated modify the rheology of the mucosal layer and deprive the mouth of its main defense, with possible serious, for the onset of even carcinogenic pathologies, (Duca *et al*., 2015).

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