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Interaction of astringent grape seed procyanidins with oral epithelial cells

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ABSTRACT

Mechanisms underlying astringency perception are not clearly understood. It has been suggested that oral epithelial cells play a role. A cell-based bioassay has been developed to define the interactions of astringent grape seed procyanidins with oral epithelial cells. Procyanidins are visualised and quantified after reaction with 4-(dimethylamino)cinnamaldehyde. This assay has demonstrated, conclusively, that astringent procyanidins bind to oral epithelial cells, and that the binding is concentration-, pH- and temperature-dependent, but is not affected by the presence of up to 13% ethanol. Furthermore, the presence of 13% ethanol did not alter *in vivo* astringency thresholds. However, a decrease in pH resulted in enhanced binding of grape seed procyanidins to oral epithelial cells, which supports our *in vivo* findings of decreased astringency recognition threshold at lower pH. These data indicate that the cells of the oral cavity may play a direct role in the astringent sensation.

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1. Introduction

Oral astringency is a sensation of roughing and drying that is familiar to most consumers of procyanidin-rich foods and beverages, and plays a primary role in the quality and mouthfeel of red wines (Gawel, 1998) and the palatability of some functional foods and therapeutics (Drewnowski & Gomez-Carneros, 2000). The flavour experienced whilst consuming wine results from a combination of taste, olfactory and tactile stimuli. Astringency is considered by some to be a taste, but the fact (amongst others) that it can be perceived when the stimulus is applied to areas of the oral cavity where there are no taste receptors lends support to the hypothesis that it is, in fact, a tactile stimulus (Green, 1993). As well as contributing to wine's flavour, the sensation also enhances complexity and length (Gawel, Iland, & Francis, 2001).

Compounds present in wine other than, for example, grape acids, which are known to be astringent, include two classes of tannin. Hydrolysable tannins pass into the wine during storage in wooden vessels or during treatment with either wood chips or exogenous tannin products. They are present at concentrations below the detection threshold of most consumers (Pocock, Sefton, & Williams, 1994) and readily degrade at wine pH (Puech, Feuillat, & Mossedale, 1999). Procyanidins, a class of condensed tannins extracted from grape seeds and skins, are relatively stable in wine (Singleton & Draper, 1964), and make a significant contribution to the mouthfeel (Gawel, 1998).

Monomeric catechin, epicatechin, and their gallates and galloforms, which are considered to be bitter, but not astringent (Peleg, Gacon, Schlich, & Noble, 1999), readily polymerise both *in planta* and during processing, forming procyanidins, with their perceived astringency increasing as the molecules increase in size (Delcour, Vandenberghe, Corten, & Dondeyne, 1984). Commercially available grape seed extracts (GSEs) are routinely added to red wine during production to enhance their structure and ageing potential.

Despite the importance of astringency to wine quality and, thus, its economic importance to the wine industry, the mechanism by which the astringent stimulus is perceived is yet to be elucidated. Research to understand the molecular basis of oral astringency, which clearly stems from the interaction between procyanidins and protein species within the oral cavity, has principally focused upon the salivary proteins. Very little information has been published regarding the interactions of procyanidin or protein–procyanidin complexes with the oral epithelium and, indeed, this may be the primary site of the astringent sensation.

A long held belief is that the primary mechanism for perception of oral astringency is delubrication of saliva. There is considerable evidence to support the statement that salivary proline-rich

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proteins (PRPs) bind procyanidins (Charlton et al., 2002). Furthermore, it has been demonstrated that the lubricating ability of saliva is decreased after incubation with tannic acid or procyanidins and removal of the resultant precipitate by centrifugation (Prinz & Lucas, 2000). As salivary lubrication decreases, friction between oral surfaces increases, and this may provide a stimulus that is perceived as astringency (Breslin, Gilmore, Beauchamp, & Green, 1993).

Increasingly, however, reports are being published which suggest that loss of salivary lubrication is not the only mechanism involved in the perception of astringency (Clifford, 1997), and it has been speculated that the sensation may also be due, at least in part, to binding of procyanidins or procyanidin–protein complexes, to cells of the oral epithelium (Green, 1993). Guinard, Pangborn, and Lewis (1986) proposed a two stage process in which salivary protein–procyanidin complexation is followed by complexes binding to the epithelial cell proteins, and suggested that this accounted for their observation that wine astringency is exacerbated with repeated ingestion. Studies have found that small procyanidins that are unable to precipitate proteins still give rise to a feeling of astringency, which lends support to the proposal that binding to the oral epithelium may have an important role in astringency perception (Kallithraka & Bakker, 1997). The finding that increasing the secretion of PRPs in Balb mice saliva by chronic isoprotenol treatment increased tolerance to tannic acid in their drinking water is somewhat at odds with the notion that a decrease in salivary lubrication is solely responsible for oral astringency (Glendinning, 1992). More recently, Kaneda, Watari, Takashio, and Okahata (2002) demonstrated that astringent tannin adsorption to lipid-coated quartz crystal increases in the presence of peptides, suggesting that tannin–protein complexes may interact with oral cells via lipid interaction. Their results also indicated that when a critical concentration of peptide is exceeded, precipitation may dominate over adsorption of the tannin–peptide complex. This could explain Glendinning's finding (1992), where the increase in mouse salivary PRP concentration was so great that it resulted in tannin–protein complex precipitation, as opposed to adsorption to oral membranes, which in turn decreased astringency and resulted in less aversion to tannin in their drinking water.

Studies in breast and lung cancer cell lines have identified a receptor for epigallocatechin-gallate as the mammalian 67 kDa laminin receptor (Tachibana, Koga, Fujimura, & Yamada, 2004), a protein also found in the extracellular matrix of oral mucosa (Hakkinen, Uitto, & Larjava, 2000). Yet more recently, de Wijk and Prinz (2005) have conducted studies showing that oral friction was not related to salivary viscosity, and demonstrated that astringency may be due to particles from salivary protein precipitation or flocculation of dead cells. Taken together, this information strongly highlights the need to examine the interactions between astringent compounds and oral epithelium as they are very likely involved in oral perception of astringency. The concept of the existence of a trigeminal receptor on oral cells involved in astringency perception is intriguing, and requires further research.

A better understanding of the physiological processes involved in the astringent response may enable specific products to be formulated for consumers who wish to benefit from the positive health benefits of consuming tannin-rich foods and beverages (Dixon, Xie, & Sharma, 2005), but who are averse to the astringent response that they elicit.

Data from our laboratory, generated using a stopped-flow spectrometer, demonstrated that GSE reacts with oral epithelial cells *in vitro*, and led to the development of a cell-based bioassay. The assay has enabled us to demonstrate that procyanidins present in wine do bind directly to oral epithelial cells, and to study the kinetics and matrix effects of that binding.

2. Materials and methods

2.1. Chemicals

GrapEx™, a 100% grape seed tannin preparation, was kindly donated by Tarac Technologies Pty Ltd. (Nuriootpa, Australia). Graptan PC grape seed tannin was sourced from Ferco Development (Saint-Montain, France); aluminium potassium sulphate, flavan-3-ols, methanol, ethanol, sulphuric acid, Tris(hydroxymethyl)aminomethane hydrocholoride (TRIS), Folin–Ciocalteu reagent, Neutral Red solution and 4-(dimethylamino)cinnamaldehyde (DMACA) were purchased from Sigma–Aldrich (St. Louis, Missouri), fetal bovine serum was supplied by Bovogen Biologicals Pty Ltd. (Melbourne, Australia); phosphate-buffered saline (PBS), Dulbecco's Modified Eagles growth medium (DMEM), Hams F12 medium, TrypLE™, glutamine, penicillin, streptomycin sulphate and amphotericin B were from Invitrogen (Carlsbad, California). HSC-2 and HO-1-N-1 cells were generously donated by TGR-Biosciences Ltd. (Adelaide, Australia).

2.2. Stopped-flow spectrometry

The interactions of procyanidins with primary human oral epithelial cells were investigated using a Pi-star 180 Stopped Flow Spectrometer (Applied Photophysics Ltd., Leatherhead, UK).

Cheek cells were harvested from human donors, under ethics approval from The University of Adelaide Ethics Committee, by gently scraping the mucosal surfaces of the cheeks with the edge of a plastic tea spoon. Donors were required to consume nothing other than water for at least 30 min prior to sample collection. Cells were immediately transferred from the collection spoon to sterile Tris-buffered saline (TBS) and held on ice for periods of no longer than 2 h until required for assay.

Cells from multiple donors were pooled, washed twice and counted using a haemocytometer, then suspended in fresh TBS at 1×10^5 cells/ml for analysis. GSE solutions were prepared in TBS as the source of procyanidins.

Experiments were performed at 28 °C, the temperature to which 15 ml of room temperature wine rose if held in the mouth for 30 s. Procyanidin solution (100 μl) was injected into the spectrometer at the same time as an equal volume of cell suspension, and the absorbance measured over time at 500 nm.

2.3. Folin–Ciocalteu assay to measure the total phenolic content of commercial grape seed extract solutions

Difficulty in obtaining pure catechin polymers led to GrapEx™ grape seed extract, a food grade preparation made from wine marc, residual solids of the winemaking process, being used as the source of procyanidins for this study. The standard Folin–Ciocalteu assay (Folin & Ciocalteu, 1927) was modified for use in 96 well multiplates to determine the phenolic content of this product.

Briefly, stock Folin–Ciocalteu (F–C) solution was diluted 1:10 with MilliQ water. Fifty microlitre of working F–C reagent were added to 10 μl of standard or test solution in the well of a multiwell plate and incubated for 5 min at room temperature, before the addition (40 μl/well) of 7.5% sodium carbonate solution. The plate was then covered, and incubated in the dark for 2 h, before the absorbance of each well was read at 740 nm in a μQuant microtitre plate reader (Biotek Instruments, Vermont, USA). Catechin was used as a standard, and the total phenolic content of the commercial grape seed extract solutions calculated from a standard curve, using Graphpad Prism software (version 3 for Windows), then expressed as catechin equivalents in gram per litre.

2.4. DMACA assay to measure the procyanidin content of commercial grape seed extract solutions

The method of Nagel and Glories (1991) was modified for use in 96 well microtitre plates. A 0.1% solution of DMACA was prepared in acidified (0.75 M H_2SO_4) methanol. Twenty microlitre of standard or test solution were incubated with 100 μl of DMACA solution for 10 min at room temperature, and the absorbance of each well was determined at 640 nm in a μQuant microtitre plate reader. Catechin was used as a standard, and the percentage of procyanidin in the GSE calculated from a standard curve, using Graphpad Prism software.

2.5. Cell culture

Two epithelial-like cell lines derived from human oral squamous cell carcinoma were employed in the study: HO-1-N-1 cells derived from buccal mucosa, and HSC-2 derived from an oral squamous cell carcinoma. The cells were grown under standard culture conditions of 5% CO_2 at 37 °C in a humidified incubator; HSC-2 in DMEM, and HO-1-N-1 in 1/1 DMEM/Ham's F12 medium, both supplemented with 10% fetal bovine serum, 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulphate, and 0.25 μg/ml of amphotericin B. Cells were dissociated with TrypLE™ Express, a recombinant trypsin replacement enzyme.

2.6. Cell viability assay

Cell viability, in response to GSE exposure, was determined by Neutral Red uptake. Neutral Red is a stain which is taken up and retained in the lysozymes of viable cells, but cannot be retained by dead or damaged cells (Borenfreund, Babich, & Martin-Alguacil, 1988). By means of this assay it is thus possible to distinguish between viable and non-viable cells. The absorbance of the wells at the end of the assay is directly proportional to the amount of viable cells present in individual wells.

Confluent monolayers of cells were washed twice with PBS pH 7.6 to remove residual growth medium. Hundred microlitre aliquots of GSE solution, which had been sterile-filtered to remove any biological contaminants, were added to wells, and the plates returned to the incubator for 20 min. Unbound GSE was removed by washing the cells three times with PBS; then 100 μl of growth medium were added to the wells, and the plates returned to the incubator for 18 h.

Ten microlitre of Neutral Red solution (0.33% in PBS) was added to each well, and the plates incubated for a further 3 h, after which time unincorporated stain was removed by washing the plates five times with PBS. Incorporated stain was released by the addition of 100 μl/well of 1% acetic acid in 50% ethanol. Plates were shaken for 20 min, the absorbance of the wells read at 540 nm on a μQuant plate reader, and data analysed using GraphPad software.

2.7. DMACA bioassay to examine procyanidin binding to cell monolayers

In the presence of sulphuric acid, DMACA reacts selectively with catechins and procyanidins to form a blue–green product that can be quantified by measuring the absorbance of the reaction mixture at 620–640 nm (Treutter, 1989), a characteristic that has been exploited in our DMACA biosassay to enable visualisation and quantification of procyanidins bound to cell monolayers.

HO-1-N-1 or HSC-2 cells were seeded into 96 well flat bottomed tissue culture plates at a density of 1×10^5 cells/well, and grown to confluence before use in an assay.

The cell monolayers were washed twice with PBS, pH 7.6, to remove residual growth medium, and test solutions were added, in triplicate, at 100 μl/well. Test solutions were prepared in PBS, at pH 3.5 and 7.6, or ethanol concentrations of 0% and 13%, and left in contact with the monolayers for varying lengths of time between 15 s and 120 min (specifically 0.25, 0.5, 1, 2, 5, 10, 20, 30, 45 and 60 min), at temperatures of 22, 28 and 37 °C.

At the end of the incubation period, unbound material was removed by washing the cell monolayers three times with PBS, pH 7.6. The plates were then blotted dry, and 20 μl of DMACA reagent (0.1% DMACA in acidified (0.75 M H_2SO_4) methanol) added to each well. After 20 min, the absorbance of the wells at 620 nm was determined in a μQuant plate reader, and resultant data analysed using GraphPad software.

2.8. Determination of GSE astringency recognition threshold

Astringency recognition thresholds were determined *in vivo* by means of a modified half tongue test (Scharbert, Holzmann, & Hofmann, 2004). Subjects were first trained to detect astringency by sipping a solution of 0.7% aluminium potassium sulphate and describing the sensations that they experienced.

Stock solutions of 8 g/l of GSE, and 7 serial 1:1 dilutions, were prepared immediately prior to use, the solid being completely dissolved and diluted in room temperature potable water that had no odours or taints. Water (500 μl) was applied to one side of a subject's tongue, and 500 μl of the test solution to the other. Subjects then closed their mouths, and gently moved the tongue against their oral surfaces before recording whether or not they perceived any difference in sensation from one side of the tongue to the other and, if so, what the sensation was, and on which side of the tongue it was perceived. The test and control solutions were randomly pre-assigned to a specific side of the tongue. Solutions were assigned a random three digit code; thus subjects were unaware of the identity of the solutions being administered.

Test solutions were administered at increasing concentration, and recognition threshold defined as being the mean of the highest concentration of GSE at which astringency could not be perceived, and the lowest concentration at which it could.

The assay was validated by testing six female and four male subjects, aged between 22 and 60, recruited from within the Discipline of Wine and Horticulture at the University of Adelaide. Repeatability was ascertained by administering GSE solutions in stepwise ascending and descending concentrations, increasing concentration until recognition threshold was reached, then descending two concentrations, before ascending back to threshold concentration. Reproducibility was determined by testing subjects on three occasions.

In order to investigate the effect that pH had on the perception of astringency, GSE solutions were prepared in potable water at pH 7.0 or 3.5 (pH adjusted by addition of 3 M HCl). Control solutions were water at pH 7.0 or 3.5, as appropriate. 14 female and 25 male subjects, aged between 21 and 60, were recruited from within the Discipline of Wine and Horticulture, University of Adelaide, and their astringency recognition thresholds for the GSE were determined at pH 7.0 and at pH 3.5, using the modified half tongue test. The effect of ethanol on astringency perception was similarly investigated: control solution and diluent being potable water (pH 7.0) containing 13% ethanol.

3. Results

3.1. Total phenolic and procyanidin content of grape seed extract

The total phenolic content of the GSE used in this study was determined by means of the Folin–Ciocalteu assay, using catechin as a standard. Results show that the GSE, when compared to a

standard solution of catechin, is comprised of approximately 50% total phenolics.

The procyanidin content of the GSE was determined by means of the DMACA assay, using catechin as a standard. Results of this assay demonstrate that the extract is composed of approximately 13% catechins and procyanidins.

3.2. Cell viability assay

In order to determine whether the GSE is toxic to oral epithelial cells, sub-confluent monolayers of HSC-2 cells were exposed to a range of concentrations of the GSE for 20 min. Results showed that exposure of the cells to a solution of GSE at a concentration of 100 g/l had no significant effect upon the viability of the cells, as determined by the absence of differences in Neutral Red uptake observed between control and treated cells (control cells OD 540 nm 3.04 ± 0.30, treated cells OD 540 nm 3.09 ± 0.06).

3.3. Dose relationship of GSE binding to oral epithelial cells

DMACA may react selectively with monomeric catechins and procyanidins. As shown by the results of a representative assay (conducted at pH 7.6 and an incubation temperature of 28 °C) in Fig. 1, we confirmed that procyanidins present in GSE bind to cell monolayers in a dose-dependent fashion at concentrations between 0 and 13 g/l of catechin equivalents. However, the DMACA bioassay showed no binding of purified catechin to HSC-2 cells (Fig. 1). The highest concentration of test solution applied was dictated by the solubility of the extract. The lower limit of sensitivity of the assay (blank + 3 SD) was less than 0.01 g/l of procyanidin expressed as catechin equivalents.

3.4. Time course of GSE binding and temperature effects

The time course of procyanidin binding to the cell monolayers was examined by incubating the cells with a solution of GSE at ambient temperature (22 °C) for periods of 0.25, 0.5, 1, 2, 5, 10, 20, 30, 45 and 60 min. Fig. 2 illustrates that binding of the extract (6.5 g/l of catechin equivalents) to the cells follows a biphasic time course. The initial rate of binding is slow but, after 60 s, the rate accelerates until maximum binding is achieved at approximately 20 min.

The effect of temperature on procyanidin binding to oral epithelial cells was examined. Fig. 3 illustrates that procyanidin binding to HSC-2 cells is accelerated and accentuated when the incubation

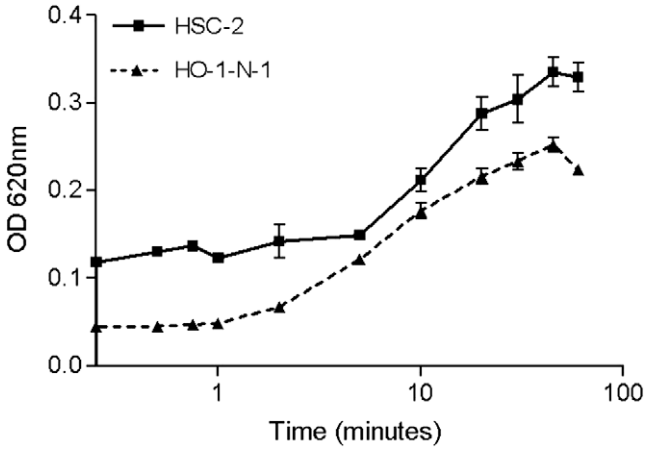

Fig. 2. Time course of procyanidin binding to HSC-2 and HO-1-N-1 cell monolayers at ambient temperature (results shown are the means and standard deviation of triplicate analyses from a representative assay).

temperature is increased from 22 to 37 °C. Binding is biphasic at all three temperatures studied (22, 28 and 37 °C), but the lag phase is shortened to less than 1 min at 37 °C. The temperature of 28 °C was chosen for investigation to mimic *in vivo* conditions. It has been observed, during trials in our laboratory (unpublished data), that when 15 ml of wine at room temperature (22 °C) is taken into the mouth and held there for 30 s, the temperature of the wine increases by 6.5 ± 0.5 °C.

3.5. Matrix effects on GSE binding

In vivo studies (De Miglio, Pickering, & Reynolds, 2002) have demonstrated that pH affects the perception of astringency. To study this effect *in vitro*, and specifically the interaction of procyanidins with oral epithelial cells, commercial grape seed extract was dissolved in PBS (pH adjusted by the addition of 3 M hydrochloric acid), and in the presence or absence of ethanol.

Fig. 4 demonstrates that procyanidin binding is significantly increased ($p < 0.05$) when the pH of the medium is decreased from 7.6 (physiological pH) to 3.5 (typical of red wines).

It has been reported that the astringent sensation is significantly affected by the presence of ethanol (Fontoin, Saucier, Teissedre, & Glories, 2008). At pH 3.5, addition of 13% ethanol to the medium had no observable effect on procyanidin binding to HSC-

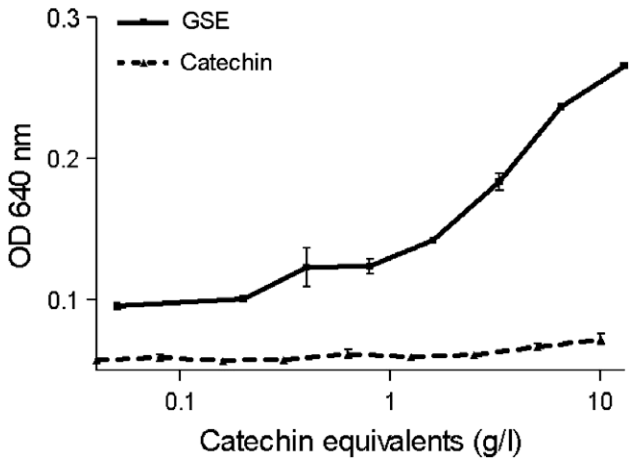

Fig. 1. Dose relationship of catechin and GSE-derived procyanidin concentration to binding to cell monolayers. Data points are means ± SD.

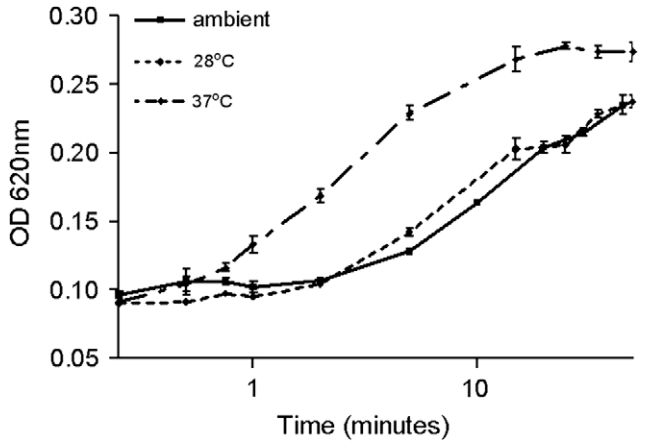

Fig. 3. The effect of temperature on the time course of commercial grape seed extract (6.5 g/l of procyanidin) binding to HSC-2 cells (means and standard deviation of triplicate analyses from a representative assay).

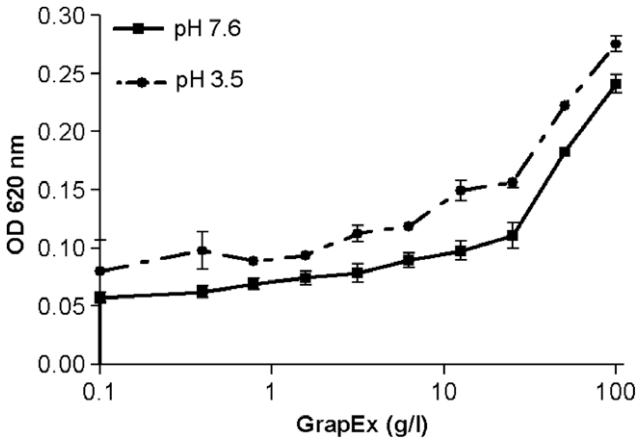

Fig. 4. The effect of pH on procyanidin binding to HSC-2 cells (results shown are the means and standard deviation of triplicate analyses from a representative assay).

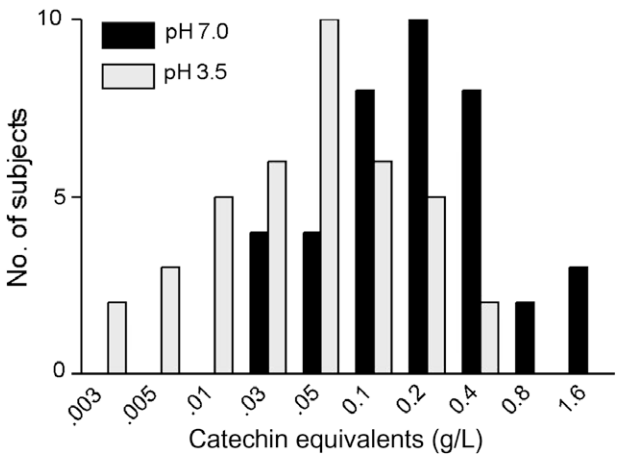

Fig. 6. Bar chart illustrating and comparing the distribution of astringency recognition thresholds for GSE at pH 7.0 and 3.5 within the sample studied, $n = 39$.

2 monolayers at concentrations typically found in red wines (Fig. 5). At concentrations above 5 g/l of GSE, binding was enhanced in the presence of 13% ethanol.

3.6. In vivo results

In order to compare the results from *in vitro* experiments with *in vivo* experiences, the recognition threshold for the astringency of commercial grape seed extract, at pH 7.0 and 3.5, was determined for 39 subjects by means of a modified half tongue test (Scharbert et al., 2004). Thresholds were also determined for 12 subjects at pH 7.0 in the presence and absence of 13% ethanol. The presence of 13% ethanol had no significant effect on astringency detection threshold ($p = 0.35$).

Fig. 6 illustrates the distribution of astringency recognition threshold, at pH 7.0 and 3.5, for the sample studied. Mean thresholds were 0.32 g/l of catechin equivalents at pH 7.0, which was reduced to 0.19 g/l of catechin equivalents at pH 3.5, demonstrating that the astringency detection threshold of the GSE is directly correlated with the pH of the solvent in which it is dissolved ($p < 0.05$).

4. Discussion

Considerable research has been conducted into interactions between tannins and salivary proteins, yet, despite suggestions that astringency may also be due, at least in part, to direct interaction between tannins and oral epithelial cells (Clifford, 1997), it has not been previously demonstrated that tannins which elicit an astringent response *in vivo* bind directly to oral epithelial cells.

Reports have been published of catechins present in green tea binding to growth factor receptors on tumour cell membranes (Tachibana et al., 2004) and endothelial cells (Lamy, Gingras, & Béliveau, 2002), and to estrogen receptors on breast tumour cells (Goodin, Fertuck, Zacharewski, & Rosengren, 2002). Procyanidins present in red wine also bind to the PDGF-ß receptor on vascular smooth muscle cells (Rosenkranz et al., 2002). That tannins bind to proteins present in skin is well evidenced by the tanning process, and it was reasonable to expect that they also bind to oral epithelial cells.

The grape seed extract used for our investigation of oral cell binding contained 50% total phenolics, as determined by the Folin–Ciocalteu assay, using catechin as a standard. Preliminary data from our laboratory, generated by means of stopped-flow spectrometry, confirmed the hypothesis that grape seed extract interacts with oral epithelial cells. However, as this procedure likely subjects the cells to considerable physical stresses, the DMACA bioassay was developed to enable study of the cell-procyanidin interaction under conditions which are less damaging to the cells.

The DMACA assay has demonstrated that procyanidins present in a commercial grape seed extract, but not monomers, such as catechin, bind to oral epithelial cells in a dose-dependent manner that is also temperature- and pH-dependent, and follows a biphasic time course.

Studies of tannin binding to salivary proteins have demonstrated that the process takes place in three stages (Jobstl, O'Connell, Fairclough, & Williamson, 2004). The first stage of the process is a reversible, weak hydrophobic attraction, followed by hydrogen bonding and cross-linking between multiple protein molecules, a process made possible by the polydentate nature of procyanidin molecules. Results from the DMACA bioassay indicate that procyanidin binding to the cells is relatively slow for the first 2 min, but then rapidly accelerates until reaching a maximum at approximately 20 min. However, the apparently slow rate of binding for the first 2 min could be an artefact of the assay procedure. Before reacting bound procyanidins with DMACA, all unbound material is removed by washing. The wash process is sufficiently gentle to ensure that the cell monolayer remains intact but could, possibly, cause any weakly, hydrophobically-bound monomers and procyanidins to be removed with unbound material. What is indisputable is that the binding process takes up to 20 min to reach

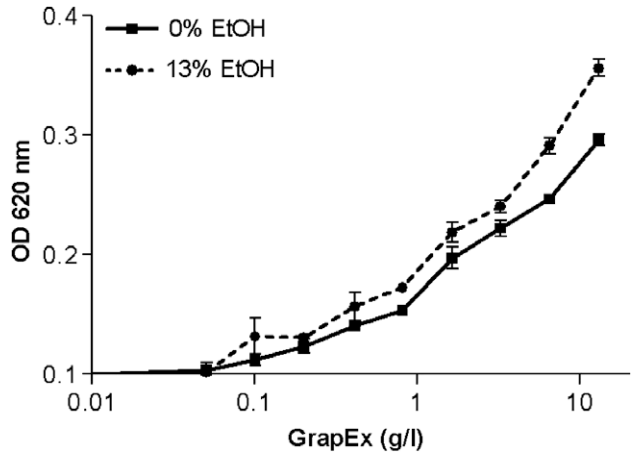

Fig. 5. The effect of 13% ethanol on procyanidin binding to HSC-2 monolayers at pH 3.5. Data points represent the means and standard deviation of triplicate analyses.

completion, and that the process is both accelerated and accentuated when the incubation temperature is increased from ambient (22 °C) to body temperature (37 °C).

The term tannin is often used to refer to a class of molecules that bind relatively non-specifically to protein molecules. It has, however, been demonstrated that procyanidins have affinities for selected proteins that differ by several orders of magnitude (Hagerman & Butler, 1981), and are dependent on a number of factors, including the isoelectric point, amino acid composition, and conformation of the protein in question, and the pH of the environment. The astringency thresholds of GSE determined in this study were well within the concentration range that also generated an astringent response in the studies conducted by Condelli, Dinnella, Cerone, Monteleone, and Bertuccioli (2006) and Parker et al. (2007). Additionally, current findings support other reports that the intensity of the astringent sensation is indirectly related to pH (Peleg, Bodine, & Noble, 1998). Enhanced astringency at low pH could be due merely to the acidic environment promoting precipitation of salivary proteins with loss of salivary lubrication. However, data from our *in vivo* studies were generated using the modified half tongue test (Scharbert et al., 2004), that measured the astringent response to a GSE test solution compared to a control solution of the same pH. Given that the subjects' astringent thresholds were lower at pH 3.5 than at pH 7.0, demonstrating a direct effect of pH, these data support the theory that the increase in perceived astringency is more likely due to an effect of pH on procyanidin binding to salivary proteins and/or oral epithelium. As it has been demonstrated that procyanidin binding to cell monolayers increased when the pH of the grape seed extract solution was decreased, the *in vitro* component of these studies supports the role of oral cells in the sensation of astringency. It has been suggested (Peleg et al., 1998) that the pH-induced shift from charged phenolate ions on the procyanidin molecules to uncharged phenolic groups that can form hydrogen bonds could be responsible for an increase in binding between protein and procyanidin molecules. In addition, procyanidins are known to have greater affinity for proteins, such as ß-casein, gelatin and the proline-rich proteins present in saliva (Jobstl, O'Connell, Fairclough, & Williamson, 2004), all of which lack tertiary structure, and have open conformations that expose multiple potential binding sites. It is possible that proteins present on membranes of oral epithelial cells become partially denatured and undergo a conformational change at low pH, with the result that more binding sites are exposed.

It is widely documented that astringency intensity is decreased as the ethanol concentration of a solution increases (Fontoin et al., 2008). Several reasons for this have been postulated, including ethanol interference with hydrogen bonding between proteins and condensed tannins (Gawel, 1998), and an ethanol induced enhanced perception of viscosity, giving rise to a decreased perception of astringency (De Miglio et al., 2002). Since the presence of 13% ethanol had no significant effect on procyanidin binding to epithelial cells in the DMACA bioassay or on *in vivo* detection thresholds determined using the modified half tongue test (Scharbert et al., 2004), the current data support the theory that the decrease in intensity of the astringent response reported in other studies is more likely related to perceived viscosity as opposed to ethanol disruption of protein–procyanidin bonding.

5. Conclusion

A cell-based bioassay has been developed and used to demonstrate that interactions between grape seed-derived procyanidins and oral epithelial cells occur. Additionally, this assay has shown that procyanidin binding to oral epithelial cells is dose-, temperature- and pH-dependent.

Use of the modified half tongue test to investigate astringency recognition thresholds has confirmed, not only that perceived astringency is indirectly related to pH, but also that the increase in perceived astringency at lower pH is, at least in part, due to a direct effect of the procyanidins, and not solely a response to the change in pH (Kallithraka, Bakker, & Clifford, 1997).

Further work is being conducted to attempt to identify specific procyanidin binding sites on the oral epithelium, to investigate binding of tannins of differing chemical structure to oral epithelial cells and to examine whether procyanidins have higher affinities for salivary proteins or for proteins present on the oral mucosa.

It is a tantalising notion that oral cells may be involved in astringency perception. Elucidation of the mechanisms will be an exciting challenge for research in this field over the next decade. Preliminary work in our laboratory has been investigating oral epithelial cell signalling post GSE binding and peripheral activation in humans observed using functional magnetic resonance imaging during ingestion of GSE.

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