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Stabilisation of collagen by betel nut polyphenols as a mechanism in oral submucous fibrosis

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Summary. Treatment of reconstituted collagen fibrils and pieces of rat dermis with the crude extract, purified tannins or (+)-catechin from betel nut *(Areca catechu)* increases their resistance to both human and bacterial collagenases in a concentration-dependent manner. These tanning agents may stabilise collagen in vivo following damage to the oral epithelium, and promote the sub-epithelial fibrosis which occurs in betel nut chewers.
Key words. Collagen; oral submucous fibrosis; tannins; flavanoids; collagenase.

Oral submucous fibrosis (OSF) is a chronic disabling disease characterised by epithelial atrophy and accumulation of collagenous scar tissue within the oral mucosa. It occurs predominantly in India, Pakistan and South East Asia and although its aetiology is obscure, OSF is closely associated with the habit of chewing betel nuts *(Areca catechu)* [1,2]. Betel nuts contain a variety of pharmacologically active compounds including arecoline, which is the most common member of a group of alkaloids responsible for the psychotropic effects of the nut. We have previously demonstrated that crude extracts from the nuts stimulate collagen synthesis by buccal mucosa fibroblasts in vitro[3] and that the alkaloids appear to be responsible for this stimulation[4]. Quantitatively, the most important group of pharmacologically active compounds are the polyphenols, which constitute more than 50% of the water-extractable compounds. These polyphenols are predominantly the flavanoid (+)-catechin, and monomeric and polymeric leucocyanidins (tannins)[5]. They are of potential importance in OSF as they can interact with proteins, conferring mechanical and chemical stability to the complexes. (+)-catechin has been shown to increase the thermal shrinkage temperature of rat skin and tendon in vitro[6], to increase the resistance of soluble collagen to degradation by mammalian collagenase[7] and to exert a protective effect against the action of lathyrogenic drugs in vivo[8].

Here we report that treatment of reconstituted collagen fibrils and rat dermal polymeric collagen with crude extracts, purified tannins or (+)-catechin from betel nuts increases their resistance to both human and bacterial collagenases.

Materials and methods. a) Preparation of betel nut extracts. 100g of raw betel nuts were powdered and defatted in petroleum ether. The residue was extracted twice in 400 ml of 70% ethanol for 2 h. Part of this extract was lyophilised and termed 'crude extract'. From the remainder, tannins were purified by the method of Wall et al.[9], briefly, an aqueous solution of the crude extract was filtered twice through celite to remove any phlobaphens, and the tannins precipitated by addition of a saturated solution of caffeine. The caffeine was removed by washing with chloroform. (+)-catechin was obtained from BDH (Poole, Dorset) and used without further purification.

b) Preparation of collagen substrates. Radioactively labeled reconstituted collagen fibrils were prepared from a solution of purified, pepsin-extracted rat skin collagen which had been ^{3}H-acetylated by the method of Gislow and McBride[10]. The collagen was dissolved in 50 mM Tris-HCl (pH 7.5), 0.165 M NaCl, 5 mM $CaCl_2$ to give a 1 mg/ml solution, sp. act. 120,000 dpm/mg. Fibrils were reconstituted from this solution by gelling at 37°C for 18 h, dispersed by vigorous shaking, and washed three times in fresh buffer. Fine dispersion was achieved by forcing the suspension through an 18-gauge hypodermic needle.

1-ml aliquots of suspended fibrils were mixed with equal volumes of crude extract, tannin and (+)-catechin solutions (1-1000 µg/ml), or buffer alone, and incubated for 18 h at 37°C. The fibrils were washed three times with buffer and exposed to either bacterial or human collagenase (see below).

Fragments of rat dermis were prepared by scraping the epidermis, fat and muscle from frozen rat skins, and mincing with scalpels to a fragment size of approximately 1 mm^3. This tissue was washed twice for 2 h at 4°C in buffer (as above). Aliquots of approximately 100 mg wet wt were incubated in 5-ml volumes of tannin solutions (1, 10 and 100 µg/ml) or in buffer alone for 18 h at 37°C. The fragments were rinsed three times in buffer before exposure to bacterial collagenase.

c) Degradation with collagenase. Triplicate 50-µl aliquots of reconstituted ^{3}H-labeled collagen fibrils were incubated at 37°C with either a) bacterial collagenase (Sigma Type II, purified by the method of Peterkofsky and Diegelmann[11]) at a final concentration of 1 mg/ml until complete dissolution of the control (untanned) fibrils had occurred, or b) human collagenase from tissue cultures of dental cyst capsule. The latent collagenase in the culture medium (Eagle's Minimal Essential Medium supplemented with 10% foetal bovine serum) was activated by incubation with 0.2% trypsin for 20 min at 25°C, and the trypsin subsequently inactivated with a 3-fold excess of soybean trypsin inhibitor (Sigma), 100 µl of this activated collagenase preparation was added to each of the collagen fibril aliquots and incubated for 5 h at 37°C. Controls contained buffer or fresh culture medium with trypsin and trypsin inhibitor in place of the collagenase preparation. After incubation the samples were centrifuged (10,000 × g for 5 min), the supernatants mixed with 3 ml Unisolve 1 scintillation cocktail (Koch Light, Colne, Middlesex), and radioactivity measured on a LKB 'Rackbeta' with external standardisation. Collagen lysis was calculated from the radioactivity released into the supernatant and expressed as the mean percentage of the total radioactivity in the tubes.

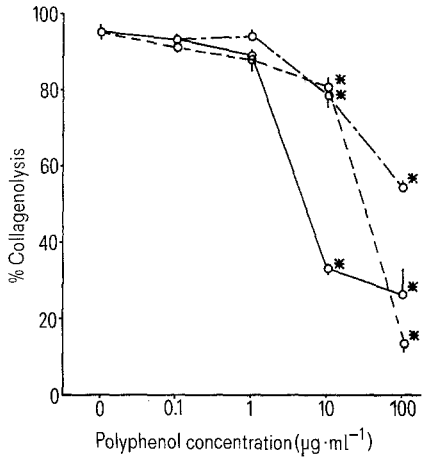

Figure 1. The activity of bacterial collagenase against ^{3}H-acetylated collagen pretreated with (+)-catechin (O—O), purified tannins (O——O) or crude extract (O·—·—·O) from betel nuts. * Significant difference from control values, $p < 0.05$.

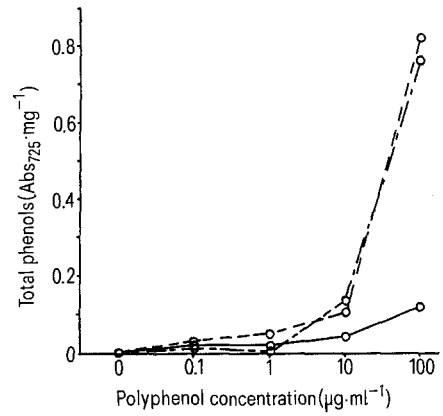

Figure 3. Total phenols in collagen fibrils preincubated with crude extract (O·—·—·O), purified tannins (O——O) or (+)-catechin (O—O) from betel nuts. All SDs were less than 0.01.

Duplicate aliquots (approximately 25 mg) of the rat dermis preparations were incubated in 1 ml of purified bacterial collagenase (as described above) at 1 mg/ml for 6 h at 37 °C. The undigested material and the supernatants were hydrolysed separately at 110 °C for 18 h in 6 N HCl, dried, and analysed colorimetrically for hydroxyproline[12]. Collagen lysis was expressed as the percentage of hydroxyproline released from the tissue into solution.

d) Analysis of total phenols. The samples were analysed for total phenols by a modification of the method of Swain and Hillis[13]. Triplicate 100-μl aliquots of the solutions were mixed with 1.0 ml Folin-Dennis reagent and 1.0 ml saturated Na_2CO_3. After 1 h at room temperature, 1.0 ml H_2O was added to each tube and the absorbance measured at 725 nm against a blank of water and reagents only. In order to measure total phenols in tanned collagen fibrils or pieces of rat dermis, the samples were heated at 100 °C for 10 min to denature them and then exposed to 3 mg/ml crude bacterial collagenase (Sigma, Type II) at 37 °C until completely lysed. The samples, including a control of bacterial collagenase alone, were then assayed for total phenols as described above. The results are expressed as absorbance units (725 nm).

In order to assess the extent to which tannins and polyphenols were extracted from betel nuts during chewing, three volunteers gave samples of saliva, chewed a small amount of betel nut (2 g) for 5 min and then gave another sample of saliva. The samples were then centrifuged at 2000 rpm for 10 min and then assayed for total phenols as described above. The results are expressed as (+)-catechin equivalents.

Results. Exposure of the reconstituted collagen fibrils to the crude extract, purified tannins and (+)-catechin of betel nuts resulted in a concentration-dependent inhibition of collagenlysis by purified bacterial collagenase (fig. 1) and by human collagenase (fig. 2). Fibrils treated with the betel nut compounds at a concentration of 1 mg/ml clumped and could not be resuspended for assay. Inhibition of lysis by both collagenases was virtually total at 100 μg/ml of each tanning agent.

Analysis of the polyphenol content of the fibrils treated revealed that they had removed these from solution (fig. 3). Although we were unable to quantitate the polyphenols bound to the collagen when applied at concentrations of 10 μg/ml or lower, analysis of the supernatants showed that all detectable phenols had been removed from solution by the collagen (results not shown).

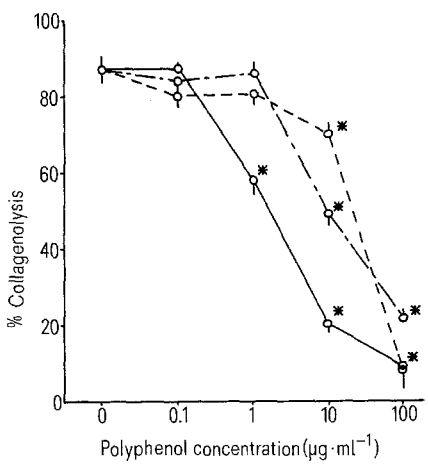

Figure 2. The activity of human collagenase against ^{3}H-acetylated collagen pretreated with (+)-catechin (O—O), purified tannins (O——O) and crude extract (O·—·—·O) from *A. catechu*. *Significant difference from control values, $p < 0.05$).

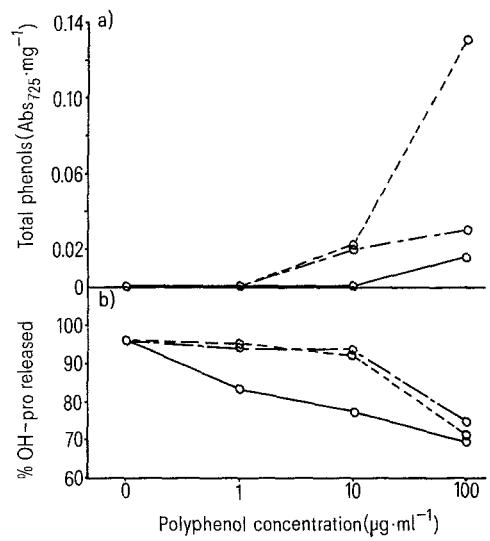

Figure 4. *a* Total phenols in pieces of rat dermis pretreated with (+)-catechin (O—O), purified tannins (O——O) and crude extract (O·—·—·O) from betel nuts. Results are expressed as absorbance units (725 nm)/mg and corrected to allow for endogenous tissue phenols. *b* The activity of bacterial collagenase against pieces of rat dermis pretreated with (+)-catechin (O—O), purified tannins (O——O) or crude extract (O·—·—·O) from betel nuts.

Exposure of polymeric collagen, in the form of fragments of rat dermis, to the nut extracts also caused a concentration-dependent inhibition of collagenolysis by purified bacterial collagenase (fig. 4b). Furthermore, this was also accompanied by an uptake of the tanning agents from the extract solutions, measured as phenol (fig. 4a).

Chewing betel nuts released polyphenols into the saliva. After 5 min chewing, the total phenols present in the saliva rose from 60–200 μg/ml to as high as 720 μg/ml (table).

Total phenols in saliva before and after chewing *Areca* nuts, expressed as catechin equivalents (μg/ml ± SD)

Subject	Before chewing	After chewing
A.S.	193.6 ± 6.9	417.1 ± 6.9
N.M.	125.9 ± 3.0	719.6 ± 0.9
S.M.	65.1 ± 4.3	695.7 ± 1.7

Discussion. We have demonstrated that the flavanoid (+)-catechin and tannins from betel nuts stabilise collagen, in the form of both reconstituted fibrils and mature polymeric collagen, to enzymatic degradation in vitro. The fact that these compounds inhibit bacterial and human collagenase, two different enzymes with different cleavage sites, suggests that this reflects a general stabilisation of collagen molecules. It is possible that the polyphenols form cross-links between the peptide chains rendering the cleavage sites inaccessable to collagenase. Harris and Farrell[14] showed that the introduction of cross-links with glutaraldehyde and formaldehyde produced a similar inhibition of collagenolysis by purified bacterial collagenase. A major difference between the action of these aldehydes and the betel nut polyphenols is that the former give rise to covalent methylene cross-links. SDS Polyacrylamide electrophoresis of the tannin-treated reconstituted fibril preparations (not shown) indicated that the polyphenols did not give rise to any new covalent bonds between the peptides. The loss of stabilising interactions between the collagen chains by denaturation in SDS and urea suggests that the polyphenols formed hydrogen bonds between the peptide chains, as proposed for (+)-catechin by Schlebusch and Kern[15]. Betel nuts are a rich source of both tannins and (+)-catechin[5]. We have shown that these are extracted into the saliva when the nut is chewed and that the concentrations achieved are well in excess of those for required for total inhibition of collagenolysis in vitro. As the betel quid is retained in the mouth for long periods of time, often overnight, the oral mucosa of the chewers will be subjected to chronic exposure to these compounds. Normally the epithelium would act as an effective barrier to these compounds, preventing their accumulation in the subepithelial lamina propria. A characteristic feature of OSF, however, is epithelial dystrophy which could enhance the penetration of the tanning agents. The major component of the lamina propria is collagen, and it is likely that exposure to (+)-catechin and tannins would result in their binding with a resultant increase in resistance to collagenolysis which we have demonstrated in vitro. As the amount of collagen in any tissue is a result of the equilibrium between synthesis and degradation, an increase in resistance to collagenase would favour an accumulation. This is likely to be exacerbated by a stimulation in collagen synthesis caused by exposure of fibroblasts to the betel nut alkaloids[4].

Although OSF is undoubtedly multifactorial, there is recent evidence that sub-clinical inflammatory and fibrotic changes occur in all betel chewers. The predisposition to clinical OSF appears to be genetic: we have recently found a highly significant association of OSF with certain HLA antigens[16], including HLA DR 3. This association indicates not only a genetic predisposition to the disease but a possible immunological basis to it. An inappropriate immunological response to an antigenic stimulus may manifest itself in the chronic inflammation which precedes the fibrotic changes in the mucosa. It is likely that these inflammatory changes would enhance the permeability of the epithelium to betel nut alkaloids, flavanoids and tannins which could then alter the synthesis and breakdown, respectively, of the subepithelial collagen.

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