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Formulation and in-vivo evaluation of L-cysteine chewing gums for binding carcinogenic acetaldehyde in the saliva during smoking

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Abstract

Cigarette smoke contains toxic amounts of acetaldehyde that dissolves in saliva, posing a significant risk of developing oral, laryngeal and pharyngeal carcinomas. L-Cysteine, a non-essential amino acid, can react covalently with carcinogenic acetaldehyde to form a stable, non-toxic 2-methylthiazolidine-4-carboxylic acid. The main aim of this study was to find out whether it is possible to develop a chewing gum formulation that would contain cysteine in amounts sufficient to bind all the acetaldehyde dissolved in saliva during the smoking of one cigarette. The main variables in the development process were: (1) chemical form of cysteine (L-cysteine or L-cysteine hydrochloride), (2) the amount of the active ingredient in a gum and (3) manufacturing procedure (traditional or novel compression method). Saliva samples were taken over 2.5 minutes before smoking and since smoking was started for 2.5 minutes periods for 10 minutes. During a five minutes smoking period with a placebo chewing gum, acetaldehyde levels increased from 0 to 150–185 μM. Once smoking was stopped, the acetaldehyde levels quickly fell to levels clearly below the in-vitro mutagenic level of 50 μM. All chewing gums containing cysteine could bind almost the whole of the acetaldehyde in the saliva during smoking. However, elimination of saliva acetaldehyde during smoking does not make smoking completely harmless. Cysteine as a free base would be somewhat better than cysteine hydrochloride due to its slower dissolution rate. Both traditional and direct compression methods to prepare chewing gums can be utilized and the dose of L-cysteine required is very low (5 mg).

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Introduction

The most important aetiological factors for oral, laryngeal, pharyngeal and oesophageal cancers are tobacco smoking and consumption of alcohol (Parkin et al 1994, Kjaerheim et al 1998, Schlecht et al 1999). According to the American Cancer Society, 90% of all oral cancer patients use tobacco and 75–80% of these cancer patients consume alcohol heavily. In developed countries, the risk of oral cancer attributable to these two factors combined is estimated to be more than 80% (Rodriguez et al 2004).

Acetaldehyde is well known as one of the most toxic compounds in cigarette smoke (Smith & Hansch 2000). The concentration of acetaldehyde in cigarette smoke is more than 1000 times greater than that of aromatic hydrocarbons (PAHs) and nitrosamines (Hoffmann & Hoffmann 1997). During smoking acetaldehyde dissolves in saliva, posing significant risk for developing oral or laryngeal carcinoma (Hoffmann & Hecht 1990; Risner & Martin 1994; Salaspuro & Salaspuro 2004). Several mammalian cell culture studies have shown that acetaldehyde concentrations of 50 to 1000 μM have caused mutagenic damage such as chromosomal exchanges, aberrations, DNA-cross links, sister chromatid exchanges and it can form stable adducts with DNA (Bird et al 1982; De Raat et al 1983; Hemminki & Suni 1984). In addition to acetaldehyde being carcinogenic in animals, a number of studies conducted in the recent past have offered strong evidence that acetaldehyde is a local carcinogen in humans (Väkeväinen et al 2000). Research has shown that after alcohol ingestion Asians with genetic deficiency of aldehyde dehydrogenese (ALDH2) enzyme have 2–3 times higher concentration of acetaldehyde in their saliva compared to those with normal ALDH2 enzyme. Also, some yeasts, such as *Candida albicans,* and Gram-positive aerobic bacteria

have a capacity to produce acetaldehyde (Homann et al 2000). Despite all the carcinogenic and in-vitro mutagenic evidence, acetaldehyde is still considered as a GRAS (i.e. "generally recognized as safe") compound used e.g. as a fruit and fish preservative (Feron et al 1991; Pohanish 2002).

It has been shown that L-cysteine, a non-essential amino acid, can react covalently with carcinogenic acetaldehyde to form a stable, non-toxic 2-methylthiazolidine-4-carboxylic acid (Sprince et al 1974). In the previous studies of our research groups, lozenges were formulated to bind salivary acetaldehyde in the oral cavity during smoking and slow-release buccal tablets to bind salivary acetaldehyde in the oral cavity after alcohol ingestion (Salaspuro et al 2002; Salaspuro et al 2006).

With the arrival of nicotine chewing gum on the market in the 1980s, a medicated chewing gum opened a new field and interest in drug research. Traditional gum process is the most frequent method for manufacturing chewing gums (Morjaria et al 2004; Maggi et al 2005). Recently, researchers have focused more on direct compression methods which offer a possibility to avoid the costs of traditional chewing gum production. By a direct compression manufacturing process, the gum base together with other chewing gum ingredients can be rapidly compacted into a gum tablet using standard tablet press equipment. Compared to the traditional process the low temperature during the direct compression process protects thermal unstable active substances.

The active components of the medicated chewing gums can be absorbed through the buccal mucosa and also through the gastrointestinal tract if saliva is swallowed (Christrup et al 1990a, b). It is also an effective drug dosage form for local treatment of diseases of the oral cavity and throat (Rassing 1996). Medicated chewing gum is easy to use, it is accepted by all age groups and it can be consumed without drinking water. Compared to lozenges, chewing gums may allow for a better control of the release rate. The main reason for this probably lies in that the lozenges are accidentally and spontaneously broken by biting, which can result in a faster drug release from formulations (Rassing et al 2003).

The main aim of this study was to find out whether it is possible to develop user-friendly chewing gum formulations that would contain L-cysteine in amounts sufficient to bind all acetaldehyde formed during smoking of one cigarette, thus helping to reduce the risk of development of upper digestive tract cancers. Variables in the development process were: (1) the chemical form of cysteine (L-cysteine free base or L-cysteine hydrochloride), (2) the amount of active ingredient in a single dose and (3) the manufacturing procedure (traditional and direct compression method). The saliva volumes were measured during smoking a cigarette and chewing a placebo gum in order to establish whether chewing has an effect on acetaldehyde concentration.

Materials and methods

Preparation of chewing gums

Direct compression method

Two different formulations were prepared. One formulation (A) contained 7.7 mg of L-cysteine (Fluka BioChemika, Buchs, Switzerland) and the other (B) 10.0 mg of L-cysteine hydrochloride (Gonmisol, Spain), corresponding to 7.7 mg of L-cysteine. The original particle size of L-cysteine hydrochloride was 90–315 μm and L-cysteine was ground and fraction 90 to 315 μm was used. Pharmagum S (SPI Pharma, New Castle, USA) was used as a gum base, 95% of total weight, and lemon flavour (1.85% of total weight) (Quest International, Netherlands) was used as a flavouring agent to disguise the unappealing taste of L-cysteine. All components of these two formulations, except for magnesium stearate, were mixed for 20 min in a Turbula shaker mixer (T2C Willy A. Bachofen A6 Maschinenfabrik, Switzerland). Magnesium stearate (2% of total weight) (Ph.Eur., Merck, Darmstadt, Germany) was added to the formulations at the end of the mixing and then it was mixed for two more minutes. The total weight of chewing gum was 1080 mg. Chewing gums were compressed with an instrumented eccentric tablet machine (Korsch EK-0, Erweka Apparatebau, Germany) using flat-faced punches with a diameter of 13 mm. The applied compression force was 7–8 kN.

Traditional method

Three formulations were prepared using the traditional gum process by Fennobon Oy, Karkkila, Finland. The first formulation (C) contained 10 mg of L-cysteine hydrochloride (Gonmisol, Spain) (equivalent to 7.7 mg L-cysteine) and the second one (D) contained 6.5 mg L-cysteine hydrochloride (equivalent to 5.0 mg of L-cysteine). Original particle size of L-cysteine hydrochloride was used (mean particle size 90–315 μm). The third chewing gum formulation was a placebo (P) containing no cysteine. Each chewing gum contained gum base (24–25%) and sweeteners such as xylitol (45%) and sorbitol (19%) as the major ingredient components. The other excipiens present in small amounts in formulations were: flavours, thickener, humectant, emulsifier, artificial sweeteners, food colour and glazing agent. The total weight of chewing gum was 1080 mg. In the course of preparation, the gum base of chewing gums was heated at a temperature between 40 and 50°C for melting. After that L-cysteine hydrochloride was added along with the other components. After mixing, the homogenous chewing gum mixture was cooled, cut into squares and hardened at room temperature. The pieces were then coated with xylitol in a coating drum, and finally polished. All formulations were shaped to be similar in weight, color and size.

In-vitro dissolution test for L-cysteine

Dissolution tests were carried out with drug dose of 20 mg using the basket method described in USP 24 (Dissolutest, Prolabo, France) in 500 mL of distilled water at 37 ± 0.5°C. The speed of rotation was 50 min^{-1}. Samples of 2 mL were taken every third minute over the period of 24 min using a pump (Marlow 503S, Smith and Nephew, UK). The samples that were taken were then compensated with 2 mL of 37 ± 0.5°C distilled water. Drug concentrations were determined by the spectrophotometer method of Eid (1998). 1.5 mL of sample was transferred to a 10-mL volumetric flask to which 0.1 mL ferric sulfate solution, 0.01 M, 0.3 μl of ferrozine, 0.01 M, and 4 mL sodium perchlorate, 0.25 M, were added.

The mixture was diluted to mark with distilled water. After 15 min at room temperature, the absorbance for each sample was measured spectrophotometrically (Ultrospec II, Pharmacia LKB Nephew, UK) at a wavelength of 562 nm. The standard curves were found to be linear (for L-cysteine hydrochloride $r^2 = 0.9986$ and for L-cysteine free base $r^2 = 0.9993$) over the concentration range used (0.25–6.0 μgmL^{-1}). Using dissolution tests results times at which 85% of the L-cysteine hydrochloride and L-cysteine free base had been dissolved ($T_{85\%}$) were calculated.

In-vivo studies

Ethics

The study was approved by the coordinating Ethics Committee, Hospital District of Helsinki and Uusimaa (Finland).

The chew-out study

The in-vivo release study of L-cysteine from chewing gums was performed by chew-out study with six volunteers. Each volunteer chewed one piece of each kind of chewing gum formulation at 12–15 and 30–35 chews for one minute. The volunteers were asked to pay attention to characteristics of the formulations, such as the taste of the chewing gum and crumbing, which can have an effect on forming complete chewing gum. The crumbing is typically characteristic of directly compressed chewing gum. At the beginning of chewing, chewing gum first crumbles into small fragments. After that using tongue movements individual fragments come together to form a gum. After chewing the gum for one minute, the chewing gum was frozen overnight at –40°C and then ground in a grinder (Braun 4041, Mexico) to obtain a fine powder. The powder was shaken out in 20 mL of distilled water for five minutes. The solution was filtered through 0.2 μm filter. 0.1 mL of filtered solution was transferred to a 10-mL volumetric flask and the remaining amount of L-cysteine was analysed by the method of Eid as described previously (Eid 1998).

Effect of cysteine chewing gums on salivary acetaldehyde during smoking

The effect of chewing gums containing L-cysteine as a free base or as a hydrochloride salt was tested in five active and one habitual smoker (4 males and 2 females; mean age 32 ± 12 years). In each of the tests, one Marlboro cigarette was smoked for five minutes. During the first smoking period, volunteers chewed a placebo chewing gum. In other four smoking periods, each volunteer chewed one piece of each kind of the four chewing gum formulations at 12–15 chews/min for five minutes. After five minutes, the smoking was stopped and the chewing gum spat out.

The saliva samples were collected: (1) continually over a period of 2.5 minutes before smoking, (2) continually during 5 minutes of smoking (with samples taken during the first 2.5 minutes stored in one collection tube, and those taken during the second 2.5 minutes into another tube) and (3) continually over the period of 5 minutes immediately after smoking (with samples taken during the first 2.5 minutes, i.e. from 5–7.5 minutes after the smoking started, stored in one collection tube, and those taken during the second 2.5 minutes, i.e. from 7.5–10 minutes after the smoking started, into another tube). All volunteers were instructed not to swallow any saliva. All subjects refrained from drinking, eating or smoking half an hour before saliva collection.

Effect of chewing a placebo gum on saliva volume and acetaldehyde levels during smoking

In order to establish whether chewing has any effect on acetaldehyde concentration, six volunteers' saliva volumes were measured. To measure the salivary acetaldehyde levels, saliva samples were collected in two ways: un-stimulated (only during smoking) and during smoking mechanically stimulated with a placebo chewing gum (12–15 chews/min for five minutes). The practical experiments were carried out analogously to those mentioned in the previous section.

Measurement of salivary acetaldehyde concentrations by gas chromatography

Acetaldehyde levels were analysed by headspace gas chromatograph (Perkin Elmer, Norwalk, CT, USA) as described in Salaspuro et al (2006) with slight modification. 500 μl of saliva was immediately transferred into a headspace vial and was kept in a cold place (5–8°C) before assay for less than one hour. The gas chromatography conditions were as follows: Column 60/80 Carbopack B/5% Carbowax 20 M, 2 m × 3 mm, Supelco, Inc., Bellefonte, PA, USA), oven temperature 37°C, transfer line and detector temperature 150°C. Each measurement was made in duplicate.

Statistical analysis

Results are expressed as mean ± s.d. In-vitro dissolution test for L-cysteine was statistically analysed with Microsoft® Excel 2002 using one-way ANOVA. All in-vivo studies were statistical analysed with Microsoft® Excel 2002 using two-way ANOVA. $P < 0.05$ was considered statistically significant.

Results and discussion

Dissolution test

The results of the dissolution tests are given in Figure 1. The dissolution rate of cysteine free base ($T_{85\%}$ 9.4 ± 4.8 min) was lower than that of hydrochloride ($T_{85\%}$ 3.6 ± 2.0 min). The difference was statistically highly significant ($P < 0.001$) and it might be exploitable in the development of the cysteine chewing gum.

In-vivo studies

The chew-out study

The in-vivo release of L-cysteine from different chewing gums under various conditions is shown in Figure 2. The first overall finding is that the active ingredient both as hydrochloride salt and also as base might release fairly quickly taking into account that smoking a cigarette takes about 5 minutes.

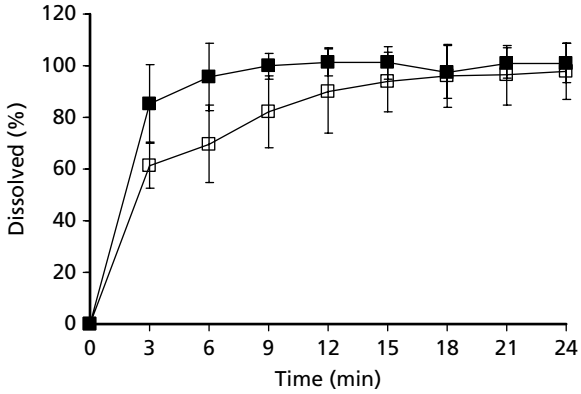

Figure 1 In-vitro dissolution profiles of L-cysteine hydrochloride (■) and L-cysteine (□) in distilled water (mean ± s.d., n = 6).

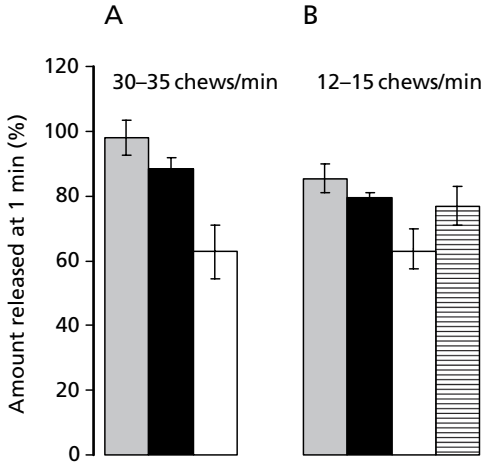

Figure 2 A. The in-vivo release of cysteine from chewing gum containing equimolar doses (7.7 mg) of cysteine when chewing frequency was 30–35 chews/min. Columns from left to right: 1. cysteine hydrochloride in directly compressed formulation, 2. cysteine in directly compressed formulation and 3. cysteine hydrochloride in traditional chewing gum. B. The in-vivo release of cysteine when chewing frequency was 12–15 chews/min from the three before mentioned formulations and from extra traditional chewing gum containing 5.0 mg of cysteine in the hydrochloride form (6.5 mg) (mean ± s.d., n = 6).

In any case, such cysteine would be a better candidate for chewing gums than the more water-soluble hydrochloride salt. The higher in-vivo release rate of the salt form is in accordance with our present in-vitro dissolution results (Figure 1). Another important finding is that the traditional chewing gum could be a better candidate for the final formulation than the directly compressed chewing gum considering that the release of cysteine was about one third slower than that of the directly compressed chewing gum (released amounts 64% and 85% for the traditionally prepared and directly compressed formulations, respectively) ($P < 0.001$). Thirdly, the results in Figure 2 reveal that a lower chewing frequency ensures more prolonged release for the active component from the directly compressed formulation ($P < 0.001$), but chewing frequency seemed to have no marked effect on drug release from the traditional chewing gum ($P > 0.05$).

The higher amount of active ingredients released at one minute from the directly compressed products can be explained by the fact that the chewing gum first broke down into smaller fragments which increase surface area and promoted a rapid initial release of the active ingredient (burst effect). Fairly quickly, however, the fragments adhered to each other forming a chewing gum using tongue movements. Thereafter the drug release can be clearly slower than in the initial phase.

According to the volunteers, the taste of the test formulations containing cysteine or its hydrochloride salt could be improved, but it was not unacceptable. Thus, even if the direct compression method offers a possibility to avoid costs caused by traditional chewing gum production, the elimination of burst effect would requires more research and development work. According to Morjaria et al (2004), the major factors in formulation of chewing gums are the nature of the gum base and manufacturing procedure.

Compared to the lozenge, cysteine containing chewing gum could be more acceptable for users. For example, in the case of dry mouth, which can be caused by a number of factors (e.g. medications and systemic diseases such as anaemia and diabetes), lozenges can stick to oral mucosa and/or tongue and can also cause some local irritation by sacking (Sreebny et al 1992; Codd & Deasy 1998). Because chewing rate affects release of cysteine, instructions on how to use the formulation must be given.

Effect of L-cysteine on salivary acetaldehyde during smoking

Over the period of five minutes of smoking, salivary acetaldehyde concentrations increased from basal level (0) to $185 \pm 27\ \mu$M with placebo and rapidly fell below the in-vitro mutagenic level (50 μM) (Figure 3). All the chewing gum formulations containing cysteine could almost totally hinder acetaldehyde's access to saliva during the first 2.5 minutes ($P < 0.001$). However, the measurable acetaldehyde levels could be found in two cases in saliva samples collected between 2.5 and 5 minutes. In these two cases, the formulation was the directly compressed formulation B (10 mg of cysteine hydrochloride) or the traditional chewing gum D (6.5 mg of cysteine hydrochloride). When smoking and chewing were stopped after 5 minutes, low acetaldehyde levels could be found from saliva in every experiment as seen in Figure 3.

The first conclusion from the results is that chewing a placebo gum in itself cannot decrease saliva acetaldehyde levels during smoking. However, with proper chewing gum formulations containing cysteine it is possible to reduce one of the tobacco smoke major toxic compounds (acetaldehyde) levels to very close to zero. However, it is not likely that the cysteine inactivates all carcinogenic substances that are found in tobacco smoke. Thus, elimination of saliva acetaldehyde during smoking does not make smoking completely harmless. Further clinical trials of the effects of cysteine on lowering the risk of development of oro-laryngeal carcinomas in smokers are needed.

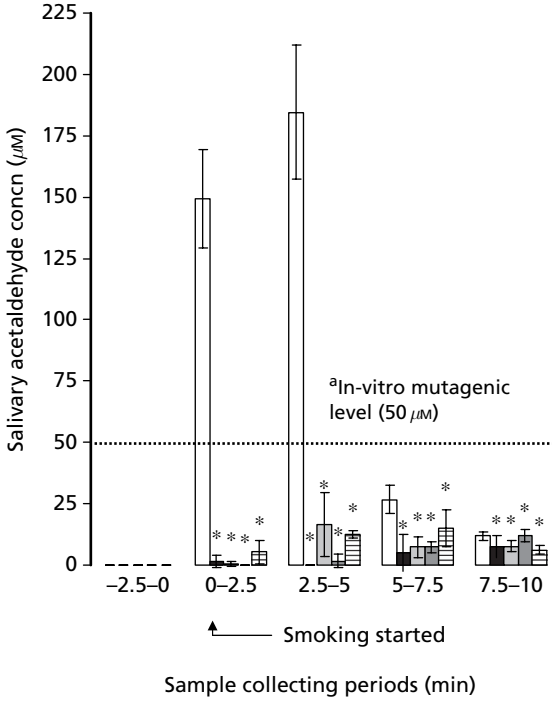

Figure 3 Effect of different chewing gums on salivary acetaldehyde levels collected in 2.5 minutes periods before and during smoking a cigarette. Columns from left to right: 1. placebo chewing gum P (□), 2. directly compressed gum A (■) (7.7 mg of cysteine), 3. directly compressed gum B (▩) (10 mg of cysteine hydrochloride), 4. traditional chewing gum C (▩) (10 mg of cysteine hydrochloride), 5. traditional chewing gum D (▤) (6.5 mg of cysteine hydrochloride) (mean ± s.d., n = 6). Smoking and chewing were stopped after 5 minutes. *$P < 0.001$ compared to placebo. [a]Mutagenic level in mammalian cell culture.

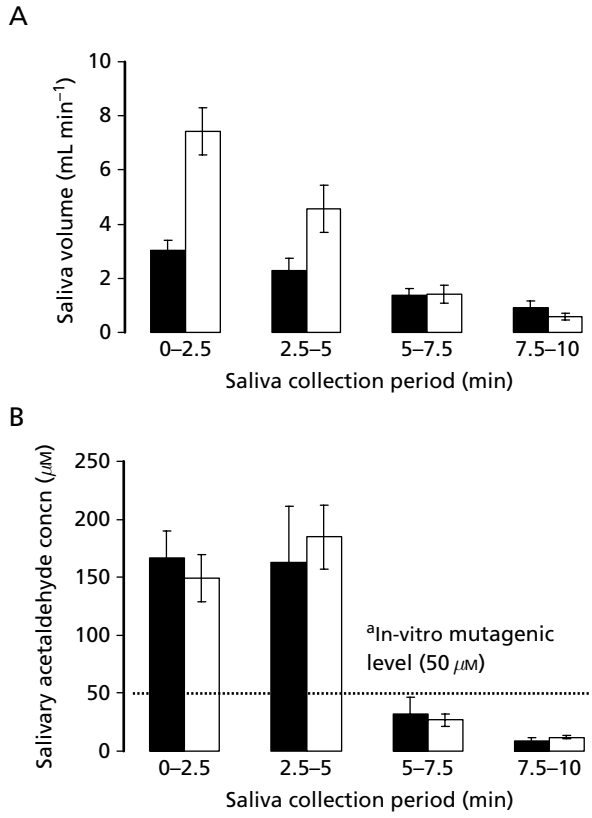

Figure 4 Changes in mean salivary volume (mL/min) (A) and corresponding changes in mean salivary acetaldehyde concentrations (μM) (B) during smoking (■) and during smoking with placebo chewing gum (□) at chewing frequency 12–15 chews/min (mean ± s.d., n = 6). Smoking and chewing were stopped after 5 minutes. [a]Mutagenic level in mammalian cell culture.

It can be concluded that cysteine in this respect is better than cysteine hydrochloride due to the slower dissolution rate of the free base. In this present study, it was obvious that the traditional chewing gum is a better dosage form than the directly compressed tablet formulation. However, if product development can succeed to hinder the temporary fragmentation of the matrix, the directly compressed chewing gum could be able to compete with traditional gums. In any case, the manufacturing procedure is much simpler than that of the traditional chewing gum.

Effect of chewing on saliva volume and acetaldehyde concentration

During the first five minutes, the mean saliva volume was 2–3 times higher in the stimulated group (Figure 4A). No statistically significant ($P > 0.05$) differences were found when unstimulated salivary acetaldehyde levels were compared with placebo stimulated salivary acetaldehyde levels (Figure 4B). The in-vivo salivary acetaldehyde levels were very high in both groups, being 184 ± 27 μM and 162 ± 23 μM for un-stimulated and stimulated group, respectively. Once the smoking was stopped and the chewing gum was removed from the mouth, there was no difference in the saliva volume and acetaldehyde levels between those two groups. Although saliva volumes markedly increased as a consequence of chewing a placebo gum, the toxic levels of acetaldehyde in saliva could not be decreased as could be expected. The amount of acetaldehyde in tobacco smoke must be very high and thus the increase in the saliva volume means only that more acetaldehyde can be dissolved. During concomitant smoking and chewing of ordinary chewing gum, there are higher amounts of carcinogenic acetaldehyde in the mouth compared to the situation that only tobacco products would be consumed. The higher the amount of acetaldehyde in the saliva, the higher is the amount of acetaldehyde that can be carried further to the oesophagus and stomach where it can induce cancer. Only chewing gums containing a component binding acetaldehyde should be recommended for concomitant use.

Conclusions

By virtue of two different manufacturing processes, we were able to prepare L-cysteine chewing gums that during the five minute smoking eliminated completely or to a substantial extent salivary in-vitro mutagenic acetaldehyde levels. The decrease in acetaldehyde levels was related to the formulation

factors, namely the amount and chemical form of the active ingredient and the manufacturing method.

The chewing gum containing L-cysteine as a free base or as hydrochloride salt can open new methods to lower the risk of development of oro-laryngeal carcinomas in smokers who fail to quit smoking. Compared to traditional manufacturing processes, the directly compressed chewing gum still needs further evaluation to achieve better acceptability by users.

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