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ORIGINAL RESEARCH ARTICLE

Transmucosal Delivery of Nicotine in Combination with Tincture of Benzoin Inhibits Apoptosis

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Abstract

Background and Objective The aim of this study was to test the hypothesis that tincture of benzoin (TOB) facilitates immediate transmucosal nicotine absorption while simultaneously promoting a safe and sustained delivery of the nicotine.

Methods In combination with TOB, nicotine toxicity and diffusion across human mucosal cells were measured using a 3-D human mucosal tissue model.

Results Nicotine was delivered 2.1 times more quickly in combination with TOB than in combination with saline ($p < 0.05$). Despite the increased diffusion, nicotine in combination with TOB significantly increased mucosal cell survival ($p < 0.05$) by reducing the release of mitochondrial cytochrome c into the cytoplasm when compared with nicotine without TOB. The average percentage distribution of cytochrome c in the cytosolic fraction over time of nicotine + 79% ethyl alcohol (ETOH) versus nicotine plus TOB (79% ETOH) was significantly different over 120 min ($60.0 \pm 29.9\%$ cytosol, $16.1 \pm 9.4\%$ cytosol, $p = 0.03$). Related to the reduction of cytochrome c release into the cytoplasm, TOB suppressed caspase-3 and -9 activity, thereby preventing intrinsic apoptosis and providing cytoprotection of the mucosal cells ($ETOH + nicotine$ vs $ETOH + nicotine + TOB$: $p = 0.008$ for caspase 3, $p < 0.001$ for caspase 9).

Conclusion Two hours of TOB (17–24% benzoin, 79% ETOH) plus nicotine promotes diffusion of nicotine across human mucosal cells and simultaneously prevents human mucosal cell toxicity by inhibiting cytochrome c release into the cytosol, thereby preventing caspase 3 and 9 activity and subsequent intrinsic apoptosis.

Key Point

Two hours of tincture of benzoin (TOB) (17–24% benzoin, 79% ethyl alcohol) plus nicotine promotes diffusion of nicotine across human mucosal cells when compared with a commercial nasal nicotine plus saline formulation, prevents human mucosal cell toxicity by inhibiting cytochrome c release into the cytosol, and this in turn prevents caspase 3 and 9 activity and subsequent intrinsic apoptosis.

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1 Introduction

Cigarette smoking accounted for at least 48% of all cancer-related deaths between 2005 and 2009 [1]. Nicotine Replacement Therapy (NRT) products for smoking cessation include nicotine-containing gums, patches, lozenges, sublingual tablets, inhalers, and mouth sprays. The most widely used NRT products are nicotine gums, and the market for these was valued at US$995.5 million in 2012. Despite its large market share, nicotine gums are clinically limited by the slow rate of nicotine transbuccal absorption

and also by their notable gastrointestinal side effects. The craving-relief effect of nicotine gum begins at the 15- to 20-min point, and as a result the quit rate for smokers using nicotine gum is reported to be as low as 3–8% within 6 months [2, 3]. The speed of onset of an NRT product is critical, with data to suggest that if acute cravings are not satisfied within 10 min, the likelihood of relapse is high [4]. Nicotine sprays and inhalers were developed to address the need for rapid onset but are beset by short-term craving relief and side effects that diminish patient satisfaction. The recent success of e-cigarettes since 2007 in the US is related to their speed of onset, minimal associated side effects, and also a perceived but undocumented safety profile relative to cigarettes.

It is difficult to study the safety profile of e-cigarettes given very few people have used e-cigarettes for an extended period of time [1]. Toxicants from e-cigarette inhalation include carcinogens such as formaldehyde, acetaldehyde, N-nitrosonornicotine (NNN), and 4(N-nitrosomethyamino)-1-(3-pyridyl)-1-butanone (NNK), and evidence exists that passive second-hand vaping of toxicants can occur [5–8]. In addition, e-cigarettes have been shown to reduce lung function, are not associated with long-term smoking cessation, and are used widely by underage consumers [1, 9–12].

The ideal NRT product, therefore, should quickly (< 10 min) reduce powerful nicotine cravings, have a prolonged effect to reduce continual product use, minimize associated nicotine side effects, eliminate inhalation and second-hand vapor/smoke, and simultaneously should not be attractive to teens. To this end, nicotine was combined with tincture of benzoin (TOB) (nicotine 1 mg/TOB 50 μL). TOB is an oral mucosal protectant that is FDA and American Dental Association approved for use in the mouth, is on the GRAS list (generally recognized as safe) as a food additive, and is a well accepted treatment for intraoral stomatitis. TOB contains 17–24% Sumatran benzoin resin solubilized in 79% ethanol. After it is applied to the mucosa, the benzoin resin forms a bioprotective film that adheres to the oral mucosa for up to 2 h.

In combination with nicotine, the TOB is hypothesized not only to facilitate immediate nicotine absorption because of its alcohol content, but also to promote a safe and sustained delivery of the nicotine. The sustained release is thought to occur because the benzoin biofilm that adheres to the mucosa is impregnated with nicotine, and the nicotine can then be released by this biofilm steadily over time. These hypotheses are tested with an in vitro transcellular diffusion model utilizing cultured human mucosal cells.

2 Methods

2.1 Nicotine Diffusion Across Human Mucosal Cells

Twenty-four-well EpiOral human mucosal cells in a 3-D mucosal model (http://www.mattek.com) were transferred from agarose to assay medium and incubated at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% relative humidity (RH) for 60 ± 5 min. Tissues were transferred to fresh medium and incubated at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH overnight (18 ± 3 h). Three tissues each were treated for 2 h at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH with either 50 μL of freshly prepared assay medium without active ingredient (negative control, pH 8.5), 50 μL commercially available nicotine nasal spray (1 mg/mL) (positive control, pH 8.5), 50 μL nicotine in vehicle without TOB (1 mg/mL nicotine, 2% peppermint, 79% ethanol, pH 8.5), 50 μL nicotine in TOB (2% peppermint, pH 8.5). The concentration of nicotine was measured with high-performance liquid chromatography (HPLC) to verify the starting concentration of each test article. Fifty microliters of medium was collected from the receptor well at 10, 20, 30, 45, 60, and 120 min. Studies were conducted over 120 min to mimic the maximum residence time of nicotine plus TOB in the oral cavity. Fifty microliters of fresh neat medium was then placed in the receptor well to replace the lost medium and to maintain contact of receptor medium with the tissue. HPLC–UV was performed on each sample. The sample was diluted for HPLC with methanol. Chromatography was performed on an Xbridge column using a gradient of acetonitrile and methanol. Nicotine was monitored at 260 nm.

2.2 Tissue Viability

To assess the tissue viability following exposure to the various samples above, the tissues were rinsed with PBS and loaded with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; mattek.com). Tissues were placed into a 24-well plate containing 300 μL of MTT (1 mg/mL) and incubated for 3 h at 37 °C, 5% CO_2. After incubation with MTT, the culture inserts were transferred into 24-well plates containing 2.0 mL of extractant (mattek.com); extraction of the MTT from the tissues was allowed to continue overnight (in the dark, at room temperature). The resulting extract was quantified by measuring the optical density (OD) at 570 nm using a UV spectrophotometer. The tissue viability was determined by normalizing to the OD for untreated tissues using the following equation: % viability = OD (treated tissue)/OD (untreated tissue) $\times$ 100.

2.3 Caspase Analysis

The Caspase-Glo® assays utilize a luminogenic caspase-9 tetrapeptide substrate (Z-LEHD-aminoluciferin) or a caspase-3/7 substrate (Z-DEVD-aminoluciferin), and a stable luciferase in buffers optimized for specific caspase activity, cell lysis, and luciferase activity. In the absence of active caspase, the caspase substrates do not act as substrates for luciferase and thus produce no light. Upon cleavage of the substrates by the respective caspase, aminoluciferin is liberated and can contribute to the generation of a luminescent signal directly proportional to the amount of caspase activity present in the sample. 24-well EpiOral human mucosal cells in a 3-D mucosal model (http://www.mattek.com) were transferred from agarose to assay medium and incubated at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH for 60 ± 5 min. Tissues were transferred to fresh medium and incubated at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH overnight (18 ± 3 h). Six tissues each were treated for 2 h at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH with either 50 μL of assay medium without active ingredient (negative control), 50 μL nicotine in vehicle without TOB (1 mg/mL nicotine, 2% peppermint, 79% ethanol, pH 8.5) (positive control), 50 μL nicotine in vehicle without TOB (1 mg/mL nicotine, 2% peppermint, 79% ethanol, pH 8.5) + 50 μM of the caspase inhibitor Z-VAD-FMK, or 50 μL nicotine in TOB (1 mg/mL nicotine, 2% peppermint, pH 8.5). The Caspase-Glo 3/7 and 9 reagents were then prepared and allowed to equilibrate to RT and mixed well. 3/7 Reagent or Caspase-Glo 9 reagent was added to three wells from each treatment group and then placed on a plate shaker at 300–500 rpm for 30 s. Incubation was performed at room temperature for 1 h. Luminescence of each sample was measured in a plate-reading luminometer. A blank reaction using the Caspase-Glo 3/7 reagent or the Caspase-Glo 9 reagent, the vehicle and cell culture medium without cells, was used to measure background luminescence associated with the cell culture system, and this value was subtracted from experimental values.

2.4 Cytochrome *c* Quantification

2.4.1 Cell Fractionation (ab109718)

24-well EpiOral human mucosal cells (http://www.mattek.com) were transferred from agarose to assay medium and incubated at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH for 60 ± 5 min. Tissues were transferred to fresh medium and incubated at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH overnight (18 ± 3 h). Eight tissues each were treated for 2 h at 37 ± 1 °C, $5 \pm 1\%$ CO_2, 95% RH with either 50 μL of assay medium without active ingredient (negative control), 50 μL nicotine in vehicle without TOB (1 mg/mL nicotine, 2% peppermint, 79% ethanol, pH 8.5), 50 μL nicotine in TOB (2% peppermint, pH 8.5). The human oral mucosal cells from each group were then washed and cytosolic extraction performed using 50 μL of extraction buffer per well for 7 min at RT. Centrifugation was performed for 3 min at $300g$ in 1.5 mL eppendorf tubes. The supernatant containing the cytosolic fraction was then removed and saved on ice, and then mitochondrial extraction was performed. Mitochondrial extraction buffer 50 μL was added to the resulting pellet for 10 min at RT with gentle agitation, and then centrifugation was performed at $300g$ for 3 min at RT. The supernatant containing the mitochondrial proteins was removed and saved on ice in a fresh labeled eppendorf tube. Nuclear extraction buffer 50 μL was then added for 10 min at RT with gentle agitation, and centrifugation repeated for 3 min at $300g$ RT. The supernatant containing the nuclear extraction was removed and saved on ice in a fresh labeled eppendorf tube.

2.4.2 Cytochrome c Protein Quantity Microplate Assay (ab110172)

Each of the mitochondrial, cytosolic or nuclear extractions was loaded into individual wells on microplates precoated with cytochrome *c* capture antibodies. A buffer control was used as a null or background reference. The microplate was incubated for 3 h at room temperature. A buffer solution was prepared by adding 15 mL $20 \times$ wash buffer (tube 3) to 285 mL deionized H_2O (wash solution). Wash solution 45 mL was then placed into a clean bottle and 5 mL of $10 \times$ blocking solution was added (incubation solution). The wells were emptied quickly by turning the plate upside down and shaking out any remaining liquid. Wash solution 300 μL was added to each well used. $20 \times$ detector antibody 1 mL was added to 20 mL of incubation solution and 200 μL of this detector solution was added to each well. The plate was incubated for 1 h at room temperature. The wells of the plate were emptied and 300 μL of wash solution added to each well used. $20 \times$ horseradish peroxidase (HRP) label 1 mL was added to 20 mL of incubation solution, the wells emptied again, and then 200 μL of this label solution was added to each well. The plate was incubated for 1 h at room temperature. The wells were emptied again and 300 μL of wash solution added to each well. This wash step was repeated four more times and the wells then emptied. Development solution 200 μL was added to each well used. The absorbance of each well was measured at 600 nm at room temperature using a spectrophotometer. A measurement was made at 15, 30, 60, and 120 min. A paired, two-tailed *t* test was performed comparing cytosolic fractions.

3 Results

3.1 Nicotine Diffusion Across Human Mucosal Cells

HPLC analysis of the concentration of nicotine across human mucosal cells indicates that in combination with saline, diffusion occurs much more slowly than in the presence of 79% concentrations of ethanol. Over time, diffusion of nicotine was accelerated by TOB in comparison with saline but reduced in comparison with ethanol without benzoin (Fig. 1).

3.2 Tissue Viability

UV spectrophotometer analysis revealed that 13% of the human mucosal cells became non-viable (87% survival rate) after 2 h of exposure to 70% ethanol, while TOB with the same concentration of ethanol did not reduce cell viability (survival rate 100%). With the addition of nicotine at 1 mg/mL to 70% ethanol, tissue viability dropped to 49% after 2 h, while the addition of TOB with the same nicotine concentration led to a 94% cell survival rate ($p < 0.05$, ANOVA). When added to saline alone at pH 8.5, nicotine at 1 mg/mL led to a 98% cell survival rate (Fig. 2).

3.3 Caspase Analysis

The cause of human mucosal cell toxicity was investigated by measuring caspase 3 and caspase 9 activity after

Tincture of Benzoin Cytoprotection of Human Mucosal Cells

Fig. 2 Tincture of benzoin (TOB) provides statistically significant cytoprotection for human mucosal cells from toxicity induced by ethanol + nicotine at 1 mg/mL after 2 h ($p < 0.05$, ANOVA). *ANOVA* analysis of variance, *ETOH* ethyl alcohol, *MTT* 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide

exposure to nicotine formulations (1 mg/mL) with and without TOB (Fig. 3). The average luminescence readings correlate with caspase activity and are shown in Table 1. The luminescence readings indicate that ETOH + nicotine activates both caspase 3 and caspase 9, and that this activation is inhibited both by Z-VAD-FMK and TOB. The difference between the means of the ETOH + nicotine luminescent readings, the ETOH + nicotine + Z-VAD-FMK, and the ETOH + nicotine + TOB readings is statistically significant (ETOH + nicotine vs ETOH + nicotine + TOB: $p = 0.008$ for caspase 3, $p < 0.001$ for caspase 9). There is no significant difference between the ETOH + nicotine + Z-VAD-FMK readings and the ETOH + nicotine + TOB readings.

3.4 Cytochrome *c* Quantification

To further investigate the cause of Caspase 3 and 9 activation and its relation to human mucosal cell toxicity, human mucosal cells were fractioned into cytosolic, mitochondrial and nuclear components after treatment with nicotine (1 mg/mL) ± TOB for 2 h. Cytochrome *c*, a well-known activator of the intrinsic pathway of apoptosis via mitochondrial release into the cytosol and secondary activation of caspase 3 and 9, was then quantified in each
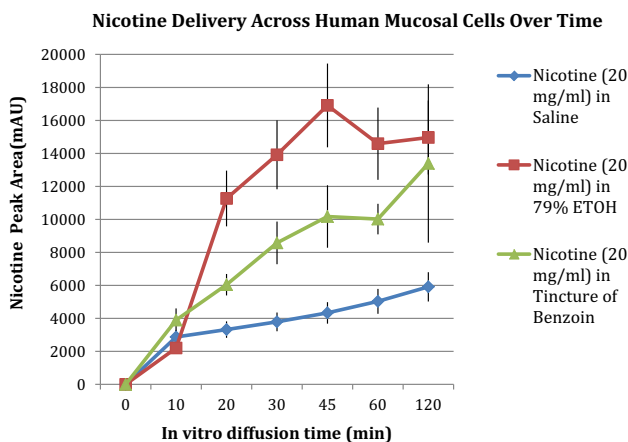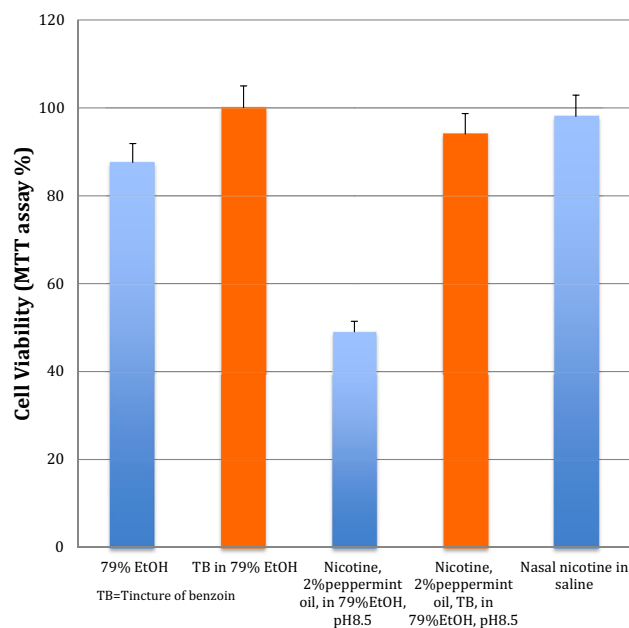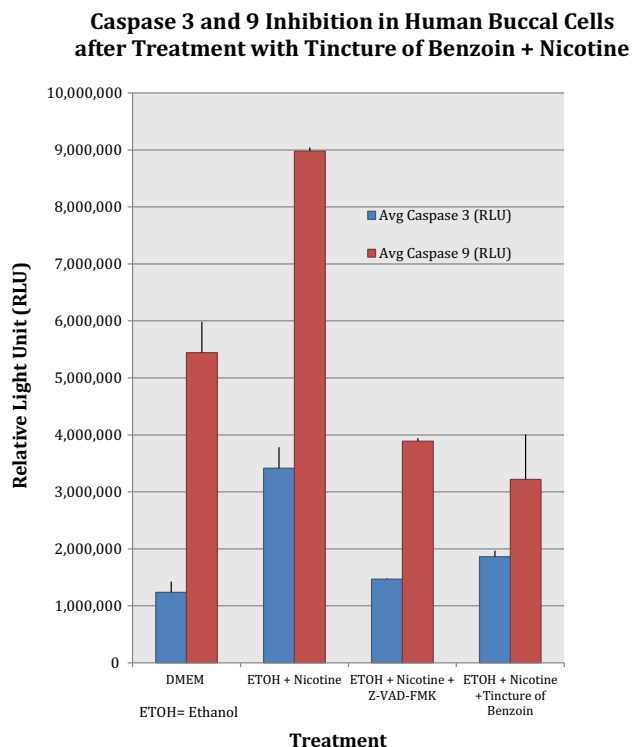

Fig. 1 Ethanol and tincture of benzoin (TOB) accelerate the delivery of nicotine across human mucosal cells in comparison with saline, whereas TOB (benzoin gum in 79% ethanol) slows the delivery of nicotine in comparison with 79% ethanol alone. Over the first 10 min, diffusion was indistinguishable, but by the 20-min point and after, nicotine in the presence of ethanol had an average diffusion rate (mAU/min) 2.1 times that of nicotine plus saline. In contrast, the diffusion rate of nicotine in the presence of TOB was on average 2.1 times that of nicotine in the presence of saline ($p < 0.05$, ANOVA). *ANOVA* analysis of variance, *ETOH* ethyl alcohol

Caspase 3 and 9 Inhibition in Human Buccal Cells after Treatment with Tincture of Benzoin + Nicotine

Fig. 3 ETOH + nicotine at 1 mg/mL triggers both caspase 3 and caspase 9 activity after 2 h, while TOB significantly reduces caspase 3 and 9 activation ($p < 0.05$, ANOVA). *ANOVA* analysis of variance, *DMEM* Dulbecco's Modified Eagle Medium, *ETOH* ethyl alcohol

fraction using a cytochrome capture antibody that is secondarily labeled with an HRP system. The average percentage distribution of cytochrome c in the cytosolic fraction over time of nicotine + 79% ETOH vs nicotine + TOB (79% ETOH) was significantly different over 120 min (60.0 ± 29.9% cytosol, 16.1 ± 9.4% cytosol, $p = 0.03$). The Dulbecco's Modified Eagle Medium (DMEM) control average was 33.9 ± 7.7% cytosol. The results indicate that ethanol + nicotine causes a shift of cytochrome c into the cytoplasm with minimal retention in the mitochondria. When nicotine is combined with TOB, however, minimal release of cytochrome c into the cytoplasm occurs until the 2-h point. The reduced release of

cytochrome c into the cytosol provides a possible explanation of the cytoprotection offered by TOB from nicotine + ETOH toxicity.

4 Discussion

4.1 In Vitro Effects of Ethanol + Nicotine on Human Mucosal Cells and the Cytoprotection Conferred by TOB

The transcellular diffusion results obtained from this study demonstrate that nicotine + 79% ethanol administration causes an increased diffusion of nicotine across human mucosal cells compared with nicotine in saline alone. These results are not surprising given the fact that ethanol as a penetration enhancer is well characterized [13–29]. Generally, ethanol is thought to increase the permeability of skin or mucosa by disrupting the lipid bilayer of the cell membrane. The mechanism of ethanol as a skin permeation enhancer is described to have a 'pull' or 'drag' effect, wherein the permeation of ethanol subsequently facilitates that of the solute (in the sense of a simple co-permeation). Bommanan et al. found that ethanol removes measurable quantities of the lipid barrier material from human skin in vivo [30]. Lipid extraction occurs and may lower the skin barrier function and render the membrane more permeable, which is the most likely explanation for the effect of ethanol as a skin penetration enhancer. Kai et al. and van der Merwe et al. published confirmatory results [31, 32]. Goates and Knutson additionally noted that the enhanced permeation caused by ethanol may be caused not only by extraction of lipids but also of proteins from human skin [33]. Nicotine in TOB (79% ethanol) showed an increase in nicotine diffusion compared with nicotine in saline but reduced compared with nicotine in 79% ethanol alone. The reduction in nicotine transport compared with ethanol alone may simply be related to mechanical stabilization of the lipid bilayer of the cell membrane by the benzoin resin, though further investigation is warranted.

Table 1 Average luminescence of human mucosal cells as an indication of caspase 3 and caspase 9 activity after 2-h exposure to nicotine formulations with and without TOB and also the caspase inhibitor Z-VAD-FMK

Test article	Avg caspase 3 (RLU)	Avg caspase 9 (RLU)
DMEM (negative control)	1,236,385 ± 10,757	5,444,720 ± 50,121
ETOH + nicotine (positive control)	3,416,346 ± 191,094	8,979,126 ± 540,531
ETOH + nicotine + Z-VAD-FMK	1,467,379 ± 368,190	3,891,464 ± 64,085
ETOH + nicotine + TOB	1,863,701 ± 102,121	3,218,476 ± 788,654

The difference between the means of the (ETOH + nicotine vs ETOH + nicotine + TOB) is statistically significant for both caspase 3 and 9 ($p < 0.05$, ANOVA)

DMEM Dulbecco's Modified Eagle Medium, *ETOH* ethyl alcohol, *RLU* Relative Light Units, *TOB* tincture of benzoin

The increased diffusion of nicotine in combination with ethanol alone correlates with a dramatic increase in human mucosal cell toxicity compared with nicotine in saline as measured by MTT analysis. However, exposing human mucosal cells to ethanol in combination with TOB leads to increased cell survival. Additionally, exposing mucosal cells to the lethal combination of nicotine and ethanol leads to 49% survival after 2 h, but when TOB is added, 94% survival takes place.

A common feature in the progression toward cell death is mitochondrial dysfunction that is associated with the release of cytochrome c from the mitochondria into the cytoplasm [34–40]. The presence of cytochrome c in the cytoplasm is often detected after a broad range of insults during acute and chronic cellular injury [37, 38, 40–42]. Once in the cytosol, cytochrome c associates with Apaf-1 to form the 'apoptosome' in conjunction with procaspase-9 which undergoes autocatalytic proteolysis to mature caspase-9. In turn, caspase-9 activates caspase-3, which in turn plays an important role in cell death [43, 44].

The toxicity induced by the ethanol + nicotine combination correlates with a dramatic increase in caspase-3 and -9 activity and release of cytochrome c into the cytosol. The addition of tincture benzoin, however, reduces caspase-3 and -9 activity and also inhibits release of cytochrome c into the cytosol. It is possible that the addition of TOB stabilizes the mucosal cell membranes, thereby preventing the ethanol + nicotine-induced release of cytochrome c into the cytosol and subsequent triggering of caspase-3 and -9. The levels of caspase-3 and -9 activity observed with the cytoprotection conferred by TOB are equivalent to the activity noted when the cells are pre-treated with the caspase inhibitor z-VAD-FMK. The cause and effect relationship between caspase-3 and -9 activation and apoptosis was demonstrated by the use of z-VAD-FMK. This inhibitor suppressed ethanol-mediated caspase-3 and -9 activation and attenuated ethanol-induced apoptosis, similar to that noted in hepatic cells exposed to ethanol [45]. As far as we know, this is the first study to demonstrate that ethanol + nicotine induces human mucosal cell apoptosis through activation of caspase-3 and -9 secondary to release of cytochrome c into the cytosol. The significant apoptosis observed in this study correlates with the toxic synergy of tobacco smoking and alcohol consumption which leads to a 40-fold increase in head and neck cancers compared with the risk of smoking or drinking alcohol alone. Given that the combination of TOB with nicotine leads to a rapid but prolonged delivery of nicotine along with a significant cytoprotective effect, further pharmacokinetic studies of this potentially beneficial transmucosal NRT product are warranted.

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