Appendix C - "Xenobiotic Modulation of the Post-Translational Modification of Collagen"
P.I. W.M. Pierce

Inhibition of Cell Metabolism by a Smokeless Tobacco Extract: Tissue and Species Specificity (43349)

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Abstract. **Smokeless tobacco contains a nonnicotine inhibitor of posttranslational modification of collagen (hydroxylation of [^{3}H]proline) by cultured chick embryo tibias and osteoblasts. This study was undertaken to determine whether a methanol extract of smokeless tobacco (STE) containing the inhibitor has similar effects on collagen-producing cells and tissues other than bone. Its effects on DNA synthesis and cell proliferation (incorporation of [^{3}H]thymidine) were also determined. Frontal bone, aorta, and cartilage were incubated for 2 days in medium containing STE. Glycolysis (lactate production) was stimulated by 80% in cartilage, but was not affected in the other tissues; medium alkaline phosphatase activity was unaffected. In frontal bone and cartilage, [^{3}H] hydroxyproline content was decreased 88% and 57%, respectively, and [^{3}H]proline content was decreased 68% and 37%, respectively; neither was affected in the aorta. Confluent cultures of collagen-producing mouse fibroblasts or primary osteoblasts obtained from chick embryo calvarias were incubated for 2 days in medium containing increasing concentrations of STE. Glycolysis and DNA synthesis were not affected. Cell proliferation was unaffected in fibroblasts, but was inhibited (34%) at the highest STE concentration in osteoblasts. AlPase activity was not detectable in fibroblast medium, but was decreased up to 72% in osteoblast medium. Inhibition of collagen synthesis by STE was concentration related in both cell types. At the highest concentration, [^{3}H] hydroxyproline and [^{3}H]proline contents in the cell layers were decreased to the following respective values: fibroblasts 56% and 45% and osteoblasts 50% and 29%, respectively. When incubation with STE was discontinued for 1 day, recovery did not occur. These findings suggest that inhibition of collagen synthesis by STE is not specific for bone, that collagen-producing cells are directly affected, and that recovery is not immediate. This inhibitor could contribute to the periodontal disease often seen in users of smokeless tobacco. Its identification and removal would produce a safer product.**

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The use of smokeless tobacco products has been strongly associated with damage to tissues of the oral cavity. Clinical studies have yielded reports of increased incidence of oral mucosal lesions, gingival and periodontal inflammation, cervical tooth erosion, gingival erosion, and cancer (1–3). With periodontosis, there is often an associated loss of underlying bone (4, 5).

A possible relationship between the use of smokeless tobacco and alveolar bone loss has been suggested in a report from this laboratory in which the effects of a potent, nonnicotine inhibitor of bone metabolism were described using a crude aqueous, smokeless tobacco (STE) extract (aqueous-STE) (6). This *in vitro* study using embryonic chick tibias treated with aqueous-STE showed a substantial inhibition of bone energy metabolism and collagen synthesis. When aqueous-STE was removed, the tibias were able to recover. More recent studies using osteoblast-like cells isolated from embryonic chick calvarias demonstrated that the addition of a methanol extract of smokeless tobacco to the culture medium markedly decreased

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alkaline phosphatase (AlPase) activity, proline hydroxylation, and collagenase-digestible protein content. Additionally, the production of collagenase-digestible protein was decreased far more by STE than was production of collagenase-indigestible protein. These characteristics were very similar to the inhibition obtained using a known prolyl hydroxylase inhibitor, 2,2′-dipyridyl (7).

These data suggest that the inhibitor in STE is specific for collagen synthesis and that the decreased collagen synthesis may result from inhibition of proline hydroxylation (6,7); however, no information is available regarding the effect of STE on collagen production in tissues other than bone or regarding its effect on DNA synthesis and cell proliferation. The present study was undertaken to determine whether STE affects other types of collagen-producing tissues and cells and whether the effects are reversible.

Methods

Extraction of Smokeless Tobacco. STE was prepared by extracting 75 g of reference moist smokeless tobacco (University of Kentucky Tobacco and Health Research Institute, Lexington, KY) in 100 ml of methanol (high performance liquid chromatography grade, Fisher). The methanol was redistilled before use and residual water was removed by adding anhydrous sodium sulfate (250 mg/ml). After stirring for 1 hr at room temperature, the tobacco extract was squeezed through cheesecloth and filtered (Whatman No. 1). Water in the extract was absorbed with sodium sulfate (250 mg/ml) to minimize the solubility of inorganic salts, and the sodium sulfate hydrate was removed by centrifugation. Methanol was removed using a rotary evaporator. Distilled water (10 ml) was added to the sample just prior to dryness and the rotary evaporation was continued to remove the final traces of methanol. The extract was reconstituted with distilled water to a volume equal to the volume prior to evaporation of the methanol and was filter sterilized using a 0.22-μm filter. An aliquot of distilled methanol was processed using the same protocol and was added to all control cultures at a concentration equal to the highest concentration of STE.

Tissue Explant Culture. Calvarias, aortas, and sternal cartilages were removed from 19-day-chick embryos (White Leghorn; Heartland Hatchery, Portland, IN) and the periosteums were removed from the frontal bones. Tissues were rinsed in a salt solution containing Hepes buffer (25 m*M* [pH 7.4]), 6.8 mg/ml of NaCl, 400 μg/ml of KCl, 200 μg/ml of $MgSO_4$, and 1 mg/ml of glucose. Culturing was performed in a defined medium consisting of degassed Eagle's minimum essential medium with Hanks' balanced salt solution without L-glutamine and $NaHCO_3$ (Whittaker Bioproducts) and containing Hepes buffer (25 m*M* [pH 7.4]), Ca (1.8 m*M*), and P_i (1.0 m*M*). This medium was supplemented with 1 mg/ml of bovine serum albumin (Fraction V), 5 mg/ml of glucose, 75 μg/ml of glycine, 292 μg/ml of glutamine, 50 μg/ml of ascorbic acid, 40 ng/ml of vitamin B_{12}, 200 ng/ml of *p*-aminobenzoic acid, 250 ng/ml of biotin, and 100 units/ml-100 μg/ml-250 ng/ml of penicillin-streptomycin-Fungizone.

To test the effects of STE on various collagen-producing tissues, L-[5-^{3}H]proline (3 μCi/ml, sp act 33 Ci/mmol, Amersham) and STE (25 μl/ml) were added to the medium. Tissues were cultured in 6-well cluster plates and were incubated for 2 days at 38°C on a rocker platform in a humidified incubator. Each well contained four frontal bones, three aortas, or three sternal cartilages in 6 ml of medium. The medium was changed after the first day and the tissues were collected after the second day. Medium samples from both days and the tissues were analyzed.

Cell Culture. Osteoblast-like cells were harvested from 17-day chick embryo calvarias by sequential collagenase digestion. The periosteums were removed from approximately 50 calvarias, 10 ml of digestion medium containing purified collagenase (375 units/ml, Sigma) and purified protease (7.5 units/ml, Sigma) were added to the frontal bones, and they were incubated for 10 min at 38°C in a shaker bath. The digestion medium and cells released during this incubation were removed and discarded. Fresh digestion medium (10 ml) was added and incubation was continued for 30 min. These cells were harvested by centrifugation and rinsed three times in 25 m*M* Hepes-buffered, Hanks' balanced salt solution (pH 7.4). This digestion step was repeated twice and the three cell isolates were pooled in growth medium consisting of Dulbecco's modified Eagle's medium (Sigma) containing 25 m*M* Hepes, 10% NuSerum IV (Collaborative Research), 292 μg/ml of glutamine, 100 μg/ml of ascorbic acid, 40 ng/ml of vitamin B_{12}, 200 ng/ml of *p*-aminobenzoic acid, 250 ng/ml of biotin, and 100 units/ml-100 μg/ml-250 ng/ml penicillin-streptomycin-Fungizone (pH 7.4). When grown in medium containing 10% NuSerum, these cells contained and released high levels of alkaline phosphatase activity, produced mineralized nodules containing Type I collagen, and showed a marked increase in cAMP content in response to parathyroid hormone. Additional details of the isolation method and the osteoblast-like phenotypic expression of these cells have been described previously (8). Collagen-secreting, embryonic mouse fibroblasts (CCL-96) were obtained from American Type Culture Collection, Rockland, MD. Six-well cluster plates were seeded with fibroblasts (1×10^6 cells/well) and 12-well cluster plates were seeded with osteoblast-like cells (4.5×10^5 cells/well). Cells were grown to confluence in growth medium. Medium volumes were 6 ml/well in the 6-well plates or 4 ml/well in the

12-well plates. The cultures were incubated on a rocker platform in a 38°C, humidified incubator.

The effects of STE on thymidine incorporation and proline hydroxylation were examined using confluent cultures of both cell types. Cells were incubated for 2 days in medium containing STE (10, 12.5, 17, or 25 μl/ml) and [^{3}H]proline (1 μCi/ml) or [*methyl*-^{3}H]thymidine (1 μCi/ml, sp act 86 Ci/mmol, Amersham). The medium was changed after the first day and the cell layers were collected after the second day. Medium from both days and the cell layers were analyzed.

To examine the ability of bone cells to recover from the effects of STE, two sets of confluent osteoblast-like cell cultures were incubated for 2 days in medium containing STE (10, 15, 25, or 30 μl/ml). The medium was changed after 1 day, and [^{3}H]proline (1 μCi/ml) was added to the medium of one set of cultures and these cells and media were collected for analysis at the end of the second day. The remaining cultures were incubated for an additional day in medium containing [^{3}H]proline, but no STE. These cells and media were collected for analysis at the end of the third day.

DNA Determination and Thymidine Incorporation. Cell layers incubated with [^{3}H]thymidine were rinsed in 150 m*M* NaCl and suspended in 1 ml of 1 *M* NaCl, 0.1% Triton X-100, and 0.01% trypsin inhibitor (soybean Type II, Sigma). Cell layers were digested with proteinase K (1 mg/tube, Sigma) at 60°C overnight. Total DNA content was determined using Hoechst 33258 fluorescent dye. A previous publication contains a detailed description of this extraction and fluorometric method (8). The solubilized cell layers were then placed in an ice bath and precipitated with an equal volume of ice-cold 10% trichloroacetic acid. The precipitates were collected on glass fiber filters and rinsed twice with ice-cold 5% trichloroacetic acid (9). The incorporation [^{3}H]thymidine was measured by liquid scintillation spectrometry.

[^{3}H]Hydroxyproline Determination. Following treatment, tissue explants were rinsed in 150 m*M* NaCl, frozen, and dried by lyophilization. Dry weights were obtained and used as a basis for analytical determinations for the tissue explant cultures. Dried tissues were hydrolyzed in 6 *N* HCl in sealed ampules at 110°C overnight. Cell layers incubated with [^{3}H]proline were rinsed in 150 m*M* NaCl and solubilized in 1 ml of 200 m*M* NaOH at 58°C overnight. An equal volume of 12 *N* HCl was added. Samples were sealed and hydrolyzed at 110°C overnight. The HCl was evaporated from all samples with air using a sample dryer (Reacti-Therm; Pierce). Dried hydrolysates were resuspended in 200 μl of 200 m*M* NaOH and amino acids were dansylated using 400 m*M* $NaHCO_3$ (2 μl/μl hydrolysate), 1 *N* acetic acid (1 μl/μl hydrolysate) and dansyl chloride (9 mg dansyl chloride/ml acetone and 6 μl/μl hydrolysate). This method has been published previously in detail (6).

Lactate and Alpase Determination. Lactate released to the culture medium was measured enzymatically (10). Medium AlPase activity was determined at pH 9.8 using *p*-nitrophenylphosphate as substrate (11). The enzymatic activity is expressed in units, with 1 unit representing 1 μmol of substrate hydrolyzed/hr at 38°C.

Statistical Analysis. A one-way analysis of variance followed by the Tukey-Kramer (HSD) multiple comparison analysis was performed on the data. Individual group standard deviations were used to construct the 95% confidence intervals. The data are presented as the mean and 95% confidence interval, with $P < 0.05$ considered significant.

Results

Effects of STE on Tissue Explants. The release of AlPase activity to the culture medium was not altered by exposure of cultured frontal bone, aorta, or cartilage to STE at 25 μl/ml. Control values (no STE) are shown in Table I. The AlPase activity released from frontal bone was at least 7-fold greater than that released from aorta or cartilage. STE did not affect lactate production by frontal bone or aorta, but lactate produced by cartilage was increased 80% when cultured with the extract (Fig. 1A). In control cultures, lactate production by aorta and cartilage was approximately 30-fold greater than that of frontal bones (Fig. 1A).

The [^{3}H]hydroxyproline content of the aorta was not affected by exposure to STE (Fig. 1B). However, STE significantly decreased ($P < 0.05$) the [^{3}H]hydroxyproline content of frontal bone and cartilage (88% and 57%, respectively; Fig. 1B). Similarly, the [^{3}H]proline incorporation by the aorta was not affected by the STE, while culturing with the extract decreased the [^{3}H] proline content of the frontal bone and cartilage (68% and 37%, respectively; Fig. 1C).

Effects of STE on Fibroblasts and Osteoblast-like Cells. No effect was observed on the total DNA content or on the lactate production by the fibroblasts and osteoblast-like cells exposed to STE. Control values (no STE) are shown in Table I. Likewise, [^{3}H]thymidine incorporation by the fibroblasts was not affected by STE and the [^{3}H]thymidine incorporated by the osteoblast-like cells was only slightly inhibited (34%) at the highest concentration of STE (25 μl/ml; Table I). Fibroblast cultures had no detectable AlPase activity (Fig. 2A). In treated osteoblast-like cultures, AlPase activity released to the medium was significantly decreased ($P < 0.05$) at all concentrations of STE. In control cultures, the [^{3}H]proline to [^{3}H]hydroxyproline ratio was 33 for the fibroblast cell layers and 3.9 for the osteoblast-like cell layers, suggesting that collagen production as percentage of total protein synthesis was much less in fibroblasts than in the osteoblasts. However, following

Table I. Values for Analytical Parameters Unaffected by STE[a]

Cell cultures				Tissue explant cultures	
Cell type	Lactate (mg/well)	DNA (μg/well)	[^{3}H]Thymidine (10^3 dpm/well)	Tissue type	AlPase activity (units/100 mg dry wt)
Osteoblast-like	1.9 ± 0.4[b]	10 ± 2	3.5 ± 0.3[c]	Frontal bone	63 ± 20
Fibroblast	3.3 ± 0.4	22 ± 7	3.2 ± 0.9	Aorta	9 ± 5
				Cartilage	7 ± 5

[a] Cells or tissue explants were cultured for 2 or 3 days as described in Methods.
[b] Values represent mean and 95% confidence interval of control groups.
[c] [^{3}H]Thymidine incorporation was decreased 34% at the highest concentration of STE (25 μl/ml), 2.4 ± 0.2, $P < 0.05$.

incubation with increasing concentrations of STE, the [^{3}H]hydroxyproline content in both cell types was decreased at concentrations ≥10 μl/ml. The [^{3}H]proline contents of the cell layers were decreased in osteoblast-like cells at concentration ≥12.5 μl/ml and in fibroblasts at concentrations ≥17 μl/ml. At the highest concentration of STE (25 μl/ml), [^{3}H]hydroxyproline content in the cell layer was decreased 56% in fibroblasts and 50% in osteoblast-like cells (Fig. 2B), while the [^{3}H]proline content in the cell layer was decreased 45% in fibroblasts and 29% in osteoblast-like cells (Fig. 2C).

Recovery of Osteoblast-like Cells from the Effects of STE. Following a 2-day exposure of osteoblast-like cells to STE, there was, as expected, a significant ($P < 0.05$) inhibition of AlPase activity released to the culture medium and of [^{3}H]hydroxyproline and [^{3}H]proline contents in the cell layer. Following a 1-day recovery period, these parameters did not return to control values (Fig. 3). The lactate production by these cell cultures remained unaffected during the treatment and recovery period.

Discussion

Previous reports from this laboratory have shown that aqueous and methanol extracts of smokeless tobacco contain an inhibitor(s) of bone collagen synthesis (6, 7). Additionally, we have reported that nicotine can inhibit collagen synthesis in osteoblast-like cells (12). However, the concentration of nicotine required to produce that inhibition was at least three times that contained in the highest concentration of STE used in the present study. Furthermore, removal of nicotine did not alter the inhibitory effect of STE on cultured chick tibias (6). Thus, it can be concluded that STE contains an inhibitor(s) of collagen synthesis other than nicotine.

In the present study, STE inhibited collagen synthesis in frontal bone and sternal cartilage from chick embryos. The extract also inhibited AlPase activity and collagen synthesis in embryonic chick osteoblast-like cells and inhibited collagen synthesis in collagen-secreting embryonic mouse fibroblasts. The latter data indicate that inhibition of collagen synthesis by the inhibitor is not specific for one species or cell type.

While the effect of the extract on AlPase activity and lactate production by the different tissue explants and cells varied, the decrease in [^{3}H]hydroxyproline and [^{3}H]proline content was consistent in all but the aorta. This exception may have resulted from the small proportion of collagen in the total protein synthesized by this tissue. Compared to the other tissue types tested, the [^{3}H]proline to [^{3}H]hydroxyproline ratio in the aorta was much higher (ratio of frontal bones, 2.87; aorta, 12.3; cartilage, 2.95). Additionally, the collagen-producing fibroblasts in this tissue are scattered among the collagenous and elastic fibers of the subendothelial layer of the tunica intima vasorum and may have had limited exposure to the STE. Furthermore, the tissue surrounding the fibroblasts may protect the cells from the STE by sequestration or inactivation of the inhibitor.

The exposure of the various collagen-producing tissues to STE did not alter the release of AlPase activity to the culture medium. This was consistent with our previous findings using cultured chick embryo tibias (6). However, the release of AlPase activity to the culture medium by control frontal bone was substantially greater than the release from control cartilage or aorta. This might be expected, since this bone contains a large percentage of osteoblasts and a high AlPase activity is characteristic of this cell. There was no detectable AlPase release from the fibroblast; however, the osteoblast-like cells released high levels of AlPase activity and this release was markedly inhibited by exposure of the cultures to STE. This inhibition suggests an effect of STE on bone synthetic function, since this enzyme is associated with bone accretion and osteoblastic activity (13). The discrepancy in the effects of the extract on AlPase activity released from frontal bone with respect to the effects on osteoblast-like cells may have been due to the large amount of AlPase activity that typically washes out of the freshly dissected bone, producing a large background activity and making changes in cell-dependent activity difficult to detect. Additionally, the isolated bone cells may be more sensitive to STE.

In previous studies, cultured chick embryo tibias treated with aqueous-STE showed increased lactate production (6); however, culturing tibias with a methanol

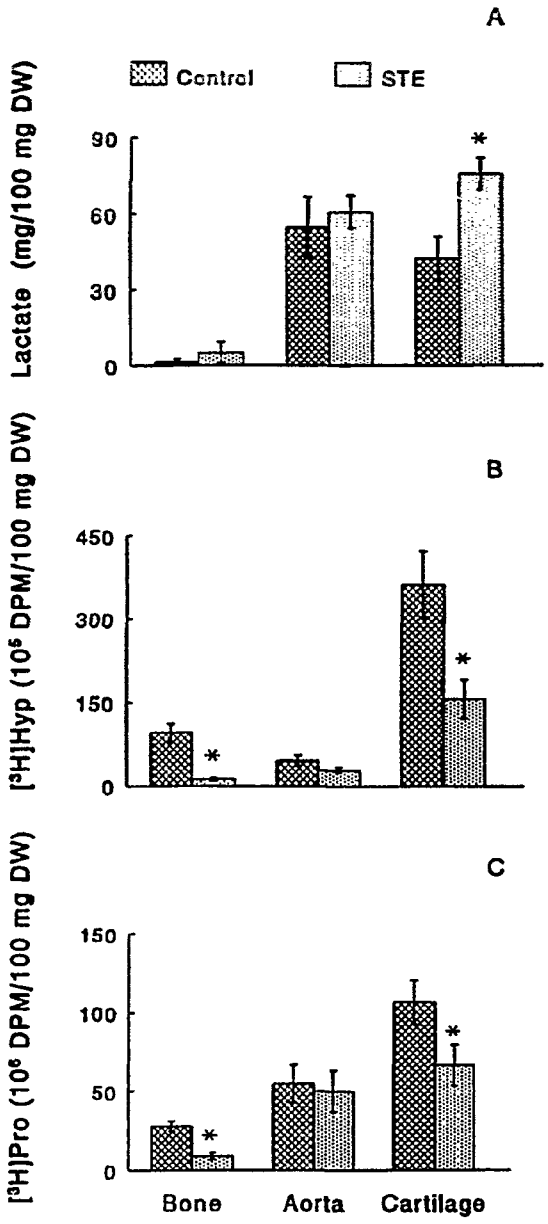

Figure 1. Effects of STE on glycolysis and collagen synthesis by cultured frontal bone, aorta, and sternal cartilage. Tissues were dissected from 19-day chick embryos and cultured for 2 days in the presence or absence of STE (25 μl/ml of medium). (A) Lactate production, (B) tissue [³H]hydroxyproline content, and (C) tissue [³H] proline content were determined. Each bar represents the mean of six wells. Vertical bars represent the 95% confidence interval. *$P < 0.05$, compared with the respective control group.

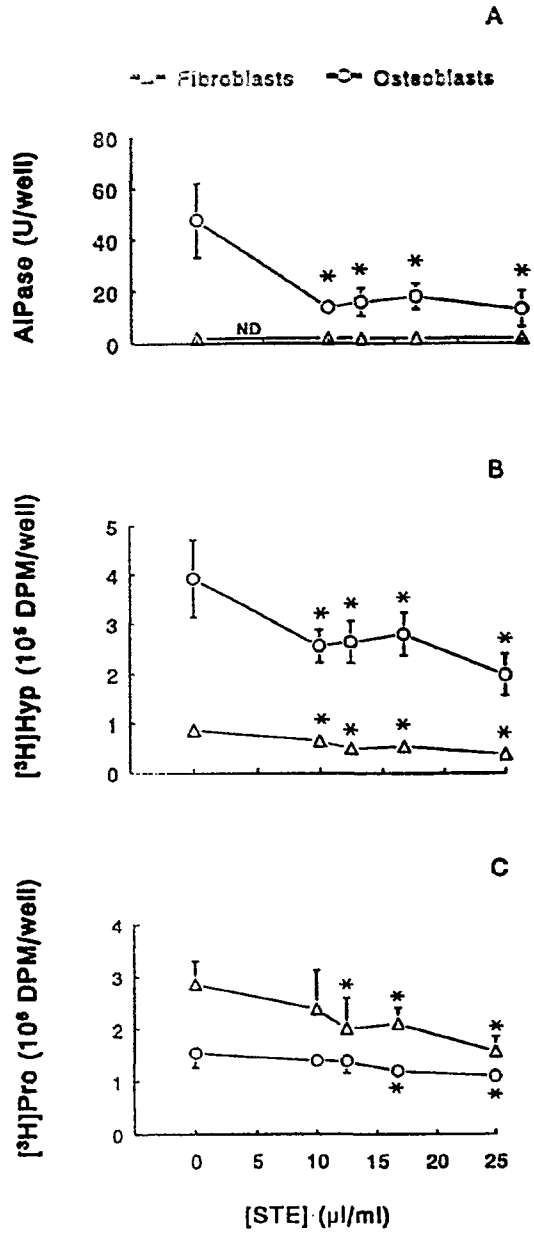

Figure 2. Inhibitory effects of STE on AlPase activity released to the culture medium and collagen synthesis by chick embryo osteoblast-like cells and mouse embryo fibroblasts. Confluent cultures were incubated for 2 days in the presence or absence of STE. The (A) AlPase activity released to the culture medium, (B) the cell layer [³H] hydroxyproline content, and (C) the cell layer [³H]proline content were determined. Each point represents the mean of six wells. Vertical bars represent the 95% confidence interval. *$P < 0.05$, compared with the control group.

extract did not produce this increase (unpublished data). In the present study, lactate production by frontal bone and aorta was unaltered by exposure to STE, while a marked increase was observed in lactate production by cartilage. Lactate production by the osteoblast-like cells and the fibroblasts was also unaltered by STE. The increase in lactate production by the cartilage exposed to STE could be the result of changes in energy metabolism. As reported previously (6), when chick tibias were incubated with aqueous-STE, a decrease in oxygen consumption (mitochondrial function) was

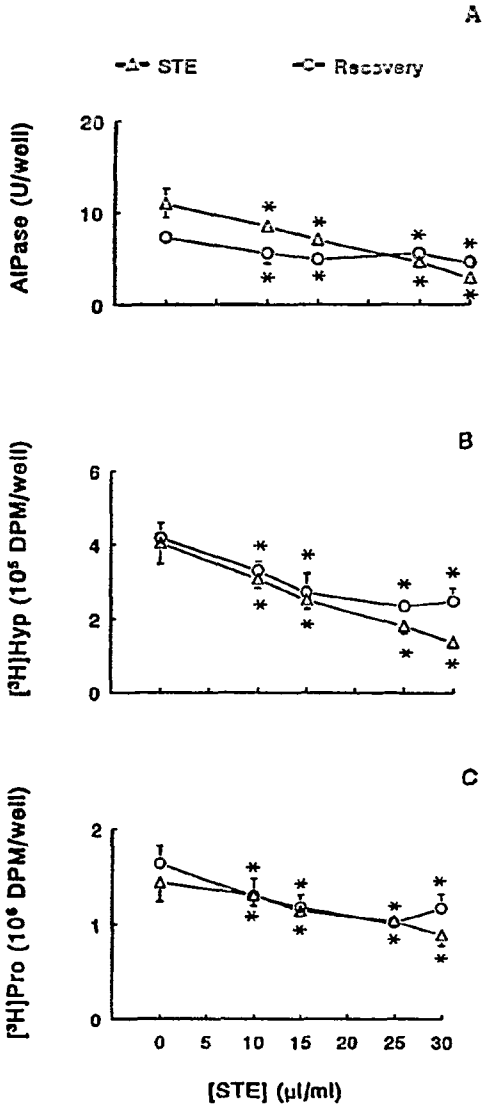

Figure 3. Test for recovery of osteoblast-like cells from the inhibition by STE. Confluent cultures were incubated for 2 days in the presence or absence of STE. At the end of the second day, the cultures were either terminated or the medium was changed to control medium and the cells were incubated for an additional day. The (A) AlPase activity released to the culture medium was determined for the second day (STE) and the third day (recovery) of the incubation period. The (B) [^{3}H]hydroxyproline and (C) [^{3}H]proline contents of the cell layer were determined following a 2-day incubation with STE and following a 1-day incubation without STE (recovery). Each point represents the mean of six wells. Vertical bars represent the 95% confidence interval. *$P < 0.05$, compared with the control group.

demonstrated concurrently with an increase in lactate production. Thus, cellular ATP requirements may have been met by increased glycolytic activity. While oxygen consumption by the cartilage treated with STE has not been tested, it might be speculated that similar changes in energy metabolism occur in this tissue. Furthermore, aqueous-STE and methanol-STE may differ in composition and their effects on glycolysis may vary. The response of the tissue may also differ according to type.

In the cell culture systems, exposure to STE did not affect total DNA content, and [^{3}H]thymidine incorporation was only slightly inhibited in the osteoblast-like cells at the highest concentration tested. This, combined with the lack of an inhibition of glycolytic activity, demonstrates that STE does not have an overtly toxic effect on the cells and does not effect cell viability.

The inhibitory effects of STE on collagen synthesis and AlPase activity released to the culture medium were not reversible during the 1-day recovery period in control medium. However, it is possible for cultured chick embryo tibias to recover from the effects of aqueous-STE during a 2-day recovery period (6). If the recovery period for the cell cultures had been increased to 2 days, some reversal of the inhibition may have occurred. Nonetheless, there was no indication of recovery at 1 day. Other contributors to this difference in results may have been differences in sensitivity of isolated cells and whole bone, and type of extraction solvent used to prepare STE.

These findings show that smokeless tobacco contains an inhibitor(s) of collagen synthesis and AlPase activity. The inhibition was acute and was observed in various collagen-producing tissues and in collagen-producing cells from both an avian and a mammalian species, thus supporting the conclusions that the inhibitor is not specific for species or tissue type and that collagen-producing cells are directly involved. Additionally, the inability of osteoblast-like cells to achieve a short-term recovery suggests that the effects may not be immediately reversible. While it is not known whether human collagen-producing cells would be affected by this inhibitor, the lack of species and tissue specificity suggests that the soft tissue damage, as well as the bone loss, seen in smokeless tobacco users may be a result of its effects. Identification and removal of this component could produce a safer product.

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1. Greer RO, Poulson TC. Oral tissue alterations associated with the use of smokeless tobacco by teen-agers. Oral Surg 56:275–284, 1983.
2. Hoffmann D, Adams JD, Lisk D, Fisenne I, Brunnemann KD. Toxic and carcinogenic agents in dry and moist snuff. JNCI 79:1281–1286, 1987.
3. NIH Consensus Development Panel. National Institutes of Health consensus statement. Health implication of smokeless tobacco use. Biomed Pharmacother 46:93–98, 1988.

4. Christen AG, Armstrong WR, McDaniel RK. Intraoral leukoplakia, abrasion, periodontal breakdown, and tooth loss in a snuff dipper. J Am Dent Assoc 98:584–585, 1979.
5. Hoge HW, Kirkham DB. Clinical management and soft tissue reconstruction of periodontal damage resulting from habitual use of snuff. J Am Dent Assoc 107:744–745, 1983.
6. Galvin RJ, Ramp WK, Lenz LG. Smokeless tobacco contains a nonnicotine inhibitor of bone metabolism. Toxicol Appl Pharmacol 96:292–300, 1988.
7. Galvin RJS, Ramp WK, Lenz LG. Comparison of the effects of smokeless tobacco extract with the effects of prolyl hydroxylase inhibitors on collagenous and noncollagenous protein synthesis by osteoblasts. Connect Tissue Res 26:217–229, 1991.
8. Ramp WK, Dillaman RM, Lenz LG, Gay DM, Roer RD, Ballard TA. A serum substitute promotes osteoblast-like phenotypic expression in cultured cells from chick calvariae. Bone Miner 15:1–17, 1991.
9. Centrella M, McCarthy TL, Canalis E. Tumor necrosis factor-α inhibits collagen synthesis and alkaline phosphatase activity independently of its effects on deoxyribonucleic acid synthesis in osteoblast-enriched bone cell cultures. Endocrinology 123:1442–1448, 1988.
10. Lundholm L, Mohme-Lundholm E, Vamos N. Lactic acid assay with L(+)lactate acid dehydrogenase from rabbit muscle. Acta Physiol Scand 58:243–249, 1963.
11. Lowry OH, Roberts NR, Wu M, Hixon WS, Crawford EJ. The quantitative histochemistry of brain II. Enzyme measurements. J Biol Chem. 207:19–37, 1954.
12. Ramp WK, Lenz LG, Galvin RJS. Nicotine inhibits collagen synthesis and alkaline phosphatase activity, but stimulates DNA synthesis in osteoblast-like cells. Proc Soc Exp Biol Med 197:36–43, 1991.
13. Rasmussen H, Bordier P. The Physiological and Cellular Basis of Metabolic Bone Disease. Baltimore: Williams & Wilkins, pp182–186, 1974.