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Cytotoxicity of Sanguinarine Chloride to Cultured Human Cells from Oral Tissue

H. Babich[1], H. L. Zuckerbraun[1], I. B. Barber[1], S. B. Babich[2] and E. Borenfreund[3]

[1]Stern College for Women, Yeshiva University, Department of Biology, 245 Lexington Avenue, New York, NY 10016, [2]New York University College of Dentistry, 345 East 24th Street, New York, NY 10016 and , [3]The Rockefeller University, Laboratory Animal Research Center, 1230 York Avenue, New York, NY 10021, U.S.A.

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Abstract: The *in vitro* cytotoxicity of sanguinarine chloride, a dental product used in the treatment of gingivitis and plaque, was compared using cell lines and primary cells from oral human tissues. For the established cell lines, sanguinarine chloride exhibited similar potencies to S-G gingival epithelial cells and to KB carcinoma cells, whereas HGF-1 gingival fibroblasts were more tolerant. However, a gingival primary cell culture was more sensitive to sanguinarine chloride than were the established cell lines. Detailed studies were performed with the S-G cells. The 24-hr midpoint (NR_{50}) cytotoxicity value towards the S-G cells was 7.6 μM, based on the neutral red cytotoxicity assay; vacuolization and multinucleation were noted. When exposed to sanguinarine chloride for 3 days, a lag in growth kinetics was first observed at 1.7 μM. Damage to the integrity of the plasma membrane was evident, as leakage of lactic acid dehydrogenase occurred during a 3 hr exposure to sanguinarine chloride at 0.1275 mM and greater. The cytotoxicity of sanguinarine chloride to the S-G cells was lessened in the presence of an S9 hepatic microsomal fraction from Aroclor-induced rats or by including fetal bovine serum (15%) in the exposure medium. Progressively increasing the pH from 6.0 to 7.8 enhanced the potency of sanguinarine chloride, presumably due to the enhanced uptake of the lipophilic alkanolamine form, as compared to that of the cationic iminium form.

Extracts of the bloodroot plant, *Sanguinaria canadensis*, have antioxidative (Firatli *et al.* 1994), antiinflammatory, and antimicrobial activities (Lenfeld *et al.* 1981). Thereby, the extracts have been clinically shown to reduce gingival inflammation and supragingival plaque (Parsons *et al.* 1987; Laster & Lobene 1990; Godowski *et al.* 1995). Sanguinaria extract or sanguinarine, the major benzophenanthridine alkaloid constituent of the extract, is an ingredient in many over-the-counter products including toothpaste, mouthwash, cough and cold remedies, and homeopathic preparations (Frankos *et al.* 1990).

Only a few isolated studies have focused on the *in vivo* toxicology of sanguinarine (Becci *et al.* 1987). There is even less information on the *in vitro* response of mammalian cells to sanguinarine. One *in vitro* study showed that the viability of human fibroblasts was not affected by sanguinaria extract at 0.003% or less (Karjalainen *et al.* 1988). The other study showed that human polymorphonuclear cell chemotaxis, chemokinesis, and adherence were inhibited by sanguinarine (1.56 to 6.21 μM) in a dose-response manner (Agarwal *et al.* 1991). The biochemical activities of sanguinarine include the inhibition of Na^+/K^+ ATPase (Straub & Carver 1975; Seifen *et al.* 1979) and of tubulin assembly (Wolff & Knipling 1993). By intercalation, sanguinarine forms a molecular complex with DNA, specifically binding to regions rich in guanosine-cytosine (Sen & Maiti 1994).

Author for correspondence: H. Babich, Stern College for Women, Department of Biology, 245 Lexington Avenue, New York, NY 10016, U.S.A. (fax 212 340 7788).

The purpose of the present research was to evaluate the *in vitro* cytotoxic response of human cell types representative of the oral cavity to sanguinarine chloride and to study various factors, i.e., metabolic activation, pH, and serum content, that may mediate the cytotoxicity of sanguinarine chloride.

Materials and Methods

Cell cultures. The human cell lines tested included Smulow-Glickman (S-G) gingival cells (obtained from F.H. Kasten, Louisiana State University Medical Center, New Orleans, LA, U.S.A.), human HGF-1 gingival fibroblasts (ATCC CRL 2014) and the KB human cell line (ATCC CCL 17) derived from an epidermoid carcinoma of the mouth, both obtained from the American Type Culture Collection (Rockville, MD, U.S.A). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone (amphotericin B) (the growth medium). Cultures were dissociated with 0.05% trypsin-0.02% EDTA. The cells were maintained in a humidified atmosphere with 5.5% CO_2 at 37°C.

Explant cultures of human gingival tissue were established as follows. Gingival tissue, obtained from a 50 yr-old healthy male, was immersed immediately in and subsequently rinsed in phosphate buffered saline (PBS) and was then placed for 1 hr in PBS supplemented with 500 U/ml polymyxin B, 500 μg/ml neomycin, and 40 U/ml bacitracin. Thereafter, the tissue was cut into 1 mm × 1 mm explants, placed in 100 mm diameter culture dishes containing 7 ml of the growth medium and maintained as above-mentioned. After about 7 days, epithelial cells and fibroblasts started outgrowth from the explants. When the primary cell culture was confluent (between 3–4 weeks), the outgrown cells (designated PGC) were detached and seeded into 96–well microtiter tissue culture plates.

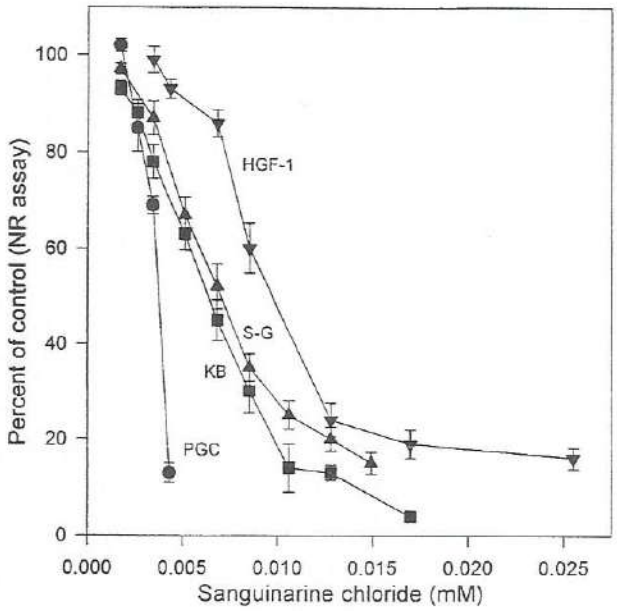


Fig. 1. Comparative responses of S-G gingival epithelial cells, HGF-1 gingival fibroblasts, KB oral carcinoma cells, and primary gingival cells (PGC) to a 24-hr exposure to sanguinarine chloride as assessed with the neutral red (NR) assay. Mean percentages of control were computed by comparisons of the NR accumulated by the treated cells to that accumulated by the control cells. Sanguinarine chloride toxicity, in mM, initially occurred ($P \leq 0.05$ or less) at 0.0034 for the PGC cells, at 0.0051 for the S-G cells, and at 0.0085 for the HGF-1 cells.

Test materials. Sanguinarine chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was solubilized in 95% ethanol. Hepatic S9 microsomal fractions from Aroclor-induced rats (Microbiological Associates, Rockville, MD, U.S.A.) were diluted with 50 mM sodium phosphate buffer to 10 mg/ml S9 protein, which was stored in 1 ml aliquots at −80° until use.

Neutral red (NR) cytotoxicity assay. Individual wells of a tissue culture 96-well microtiter plate were inoculated with 0.2 ml of the growth medium containing sufficient cells, usually 2 to 3×10^4, to provide approximately 70–80% confluence after 48 or 24 hr, respectively, of incubation. Thereafter, the growth medium was replaced with exposure medium, consisting of DMEM, 10% Serum Plus (JRH Biosciences, Lenexa, KS, U.S.A.), 2% FBS, and antibiotics, unamended and amended with varied concentrations of sanguinarine chloride. Serum Plus is a supplement, containing low levels of fetal serum proteins, enhanced with specific growth-promoting factors. Six to eight replicates were used per each treatment level. After 24 hr of exposure, cytotoxicity was assessed with the NR assay, which is based on the uptake and lysosomal accumulation of the supravital dye, NR (Borenfreund *et al.* 1990).

The protocol for the NR assay is as follows. Neutral red, as a 4 mg/ml aqueous stock solution protected from light with foil, was stored at room temperature. The exposure medium, amended to contain a working concentration of 0.04 mg/ml NR and incubated overnight at 37°, was centrifuged to remove fine dye crystals. After the appropriate time of exposure of the cells to sanguinarine chloride, the medium was removed and 0.2 ml of NR-containing medium was added to each well. Incubation of cells with NR was continued for 3 hr at 37°. Cells were then rapidly washed and fixed with 0.5% formalin-1% $CaCl_2$ (vol./vol.) and the NR incorporated into viable cells was released into the supernatant with 0.2 ml 1% acetic acid-50% ethanol. Absorbance was recorded at 540 nm with an microtiter plate spectrophotometer. Data, expressed as the arithmetic mean percentages of untreated controls ± the standard errors of the mean, were used to construct concentration-response cytotoxicity graphs. A midpoint toxicity value was reported as an NR_{50} (Borenfreund *et al.* 1990).

pH studies. Individual wells of a tissue culture 96-well microtiter plate were inoculated with 0.2 ml of the growth medium containing S-G cells to provide approximately 70–80% confluence after 48 or 24 hr of incubation. Thereafter, the growth medium was removed, the wells were washed with PBS adjusted to varied pH values, and the cells were treated for 1 hr with PBS adjusted to pH 6.0, 6.6, 7.2, and 7.8, with or without sanguinarine chloride. Thereafter, the cells were washed with PBS, refed with the growth medium, and incubated for 24 hr, after which the NR assay was performed.

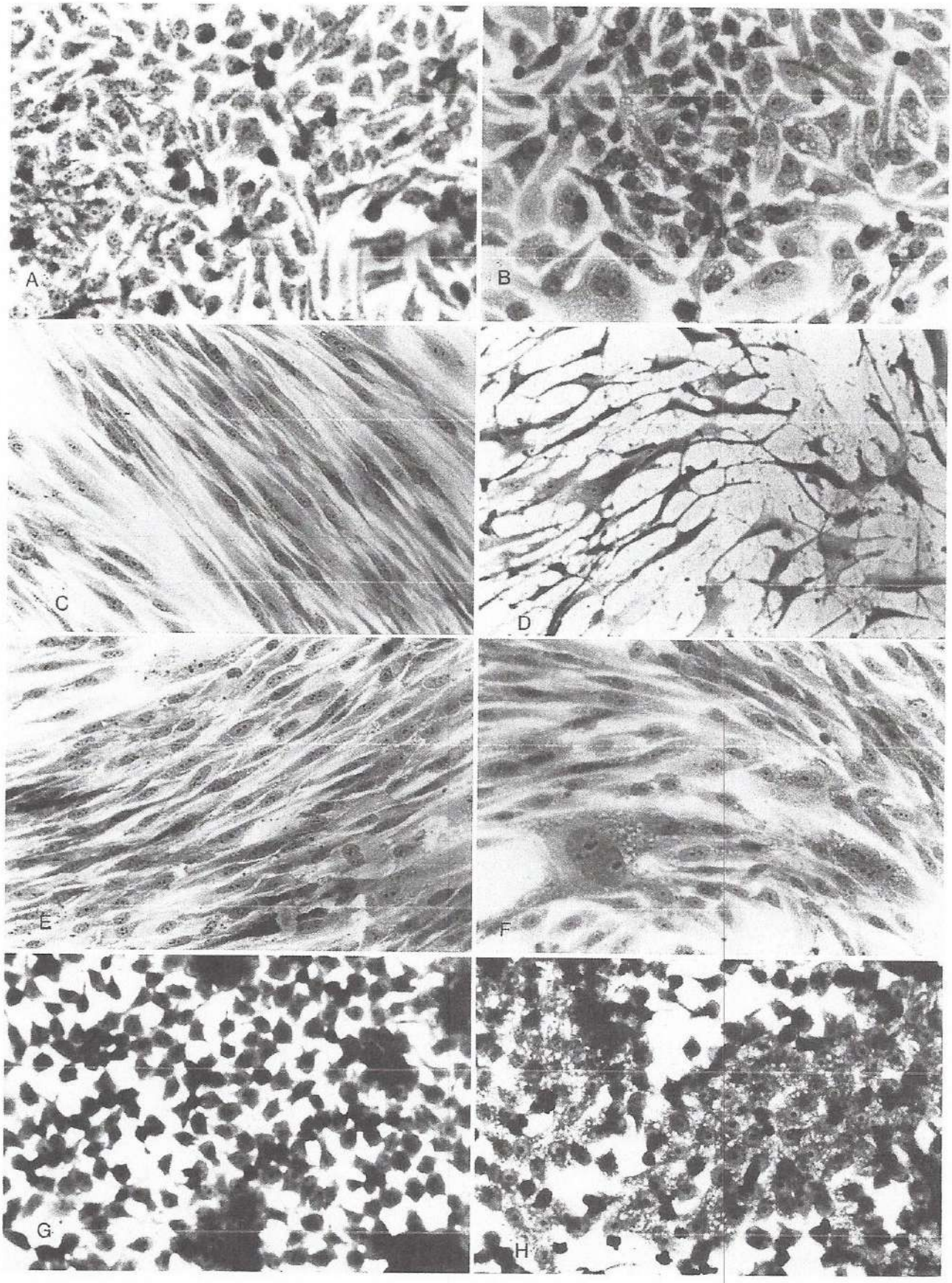
Foetal bovine serum (FBS) studies. Individual wells of 96-well microtiter plates with S-G cells at 70–80% confluence were washed with PBS and treated for 1 hr with PBS±sanguinarine, in the presence or absence of varied concentrations of FBS. Thereafter, the cells were washed with PBS, refed with the growth medium, and incubated for 24 hr, after which the NR assay was performed.

S9 studies. Individual wells of 96–well microtiter plates with S-G cells at 70–80% confluence were washed with PBS and treated for 24 hr with the exposure medium, with or without sanguinarine chloride and in the absence or presence of an activated hepatic S9 microsomal fraction, which was freshly prepared prior to each experiment. One ml of the activated S9 microsomal fraction consisted of 0.3 ml 50 mM sodium phosphate buffer (pH 7.4), 0.1 ml 100 mM $MgCl_2$, 0.1 ml 30 mM KCl, 0.1 ml 10 mM $CaCl_2$, 0.1 ml 50 mM glucose phosphate, 0.1 ml 40 mM NADP, and 0.2 ml of S9 protein preparation (10 mg/ml). For use in the NR assay, this S9 mixture was diluted 1:10 (vol./vol.) with the exposure medium, unamended or amended with sanguinarine chloride. The plates were incubated for 24 hr and thereafter the NR assay was performed (Babich *et al.* 1993).

Cell proliferation assay. The quantitative determination of DNA with the fluorochrome, bisbenzimidazole (Hoechst 33258), was used to assess the effect of sanguinarine chloride on cell proliferation. S-G cells were seeded into individual wells of a 96-well tissue culture plate and upon attaining 60% confluence, the growth medium was aspirated and the monolayer of cells was treated with the exposure medium amended with varied concentrations of sanguinarine chloride. At time zero and at 24-hr intervals for a 3 day period the plates were emptied by overturning, placed on absorbent toweling, and then stored frozen at −80°.

On the day of the assay, 0.1 ml of distilled water was added to the wells, the plates were incubated at 37° for 1 hr, refrozen at −80° for 15 min., and then thawed at room temperature to lyse the cells. Then, 0.1 ml of the fluorochrome at 0.02 mg/ml in buffer, consisting

Fig. 2. (A) S-G cells grown in control medium. Giemsa stain (magnification×260). (B) S-G cells exposed for 24 hr to 0.0051 mM sanguinarine chloride (magnification×260). (C) HGF-1 cells grown in control medium (magnification×200). (D) HGF-1 cells exposed for 24 hr to 0.0085 mM sanguinarine chloride (magnification×200). (E) Explant cells, at passage 1, in control medium (magnification×260). (F) Explant cells, at passage 1, exposed for 24 hr to 0.0026 mM sanguinarine chloride (magnification×260). (G) KB cells in control medium (magnification×260). (H) KB cells exposed for 24 hr to 0.0068 mM sanguinarine chloride (magnification×260).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of 10 mM Tris, 1 mM EDTA, and 2 M NaCl, pH 7.4, was added to each well. To ensure interaction of the fluorochrome with the DNA, the plates were briefly agitated on a rotary shaker. Thereafter, fluorescence was read with a 96–well microplate fluorometer (Cambridge Technology Inc., Watertown, MA, U.S.A.), at an excitation wavelength of 360 nm and an emission wavelength of 460 nm (Rago *et al.* 1990).

Lactic acid dehydrogenase (LDH) release. S-G cells were seeded into individual wells of a 24-well tissue culture plate and upon attaining confluence, the growth medium was aspirated, the monolayer of cells washed with PBS, and 0.5 ml of PBS, unamended and amended with varied concentrations of sanguinarine chloride, was added to each well. After a 3 hr exposure, the PBS solution (supernatant PBS) from each well was collected and stored at 4°. Cell monolayers were treated with 0.5 ml of 1 mg/ml digitonin in PBS for 30 min at room temperature to lyse the cells. LDH activity was measured in the supernatant PBS and in the cell lysate using the CytoTox 96™ Nonradioactive Cytotoxicity Assay Kit from Promega Company (Madison, WI, U.S.A.) (Sinensky *et al.* 1995).

Brightfield microscopy. Cells were seeded to alcohol-cleansed 22×22 mm glass coverslips in 35 mm diameter tissue culture dishes containing the growth medium. After a few days of incubation, the monolayers were treated with varied concentrations of sanguinarine chloride in the exposure medium for 24 hr. Thereafter, the cells were washed with PBS, fixed in 100% methanol, and stained with Giemsa's Solution (EM Science, Gibbstown, NJ, U.S.A). Cells were microscopically examined for morphological aberrations.

Statistics. The individual data points, at least constituted of quadlicates, in the graphs and tables are presented as the arithmetic means of percentage of control±standard errors of the mean (S.E.M.). Experimental data were analyzed with one- way analysis of variance (ANOVA) followed by Tukey's multiple range test for honestly significant differences (HSD). The P value of the effect had to be ≤0.05 to be considered significant.

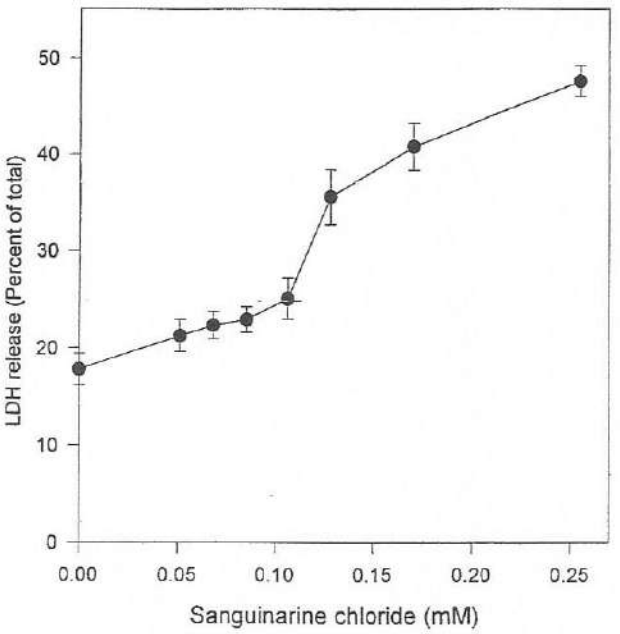


Fig. 4. LDH from S-G cells treated for 3 hr with sanguinarine chloride. Released LDH was determined as the percentage of the total LDH activity (LDH activity in PBS supernatant ÷ LDH activity in PBS supernatant + LDH activity in cell lysate). Statistically significant (P≤0.01 or less) increase in LDH release initially occurred at 0.1275 mM sanguinarine chloride.
LDH=lactic acid dehydrogenase; PBS=phosphate buffered saline.

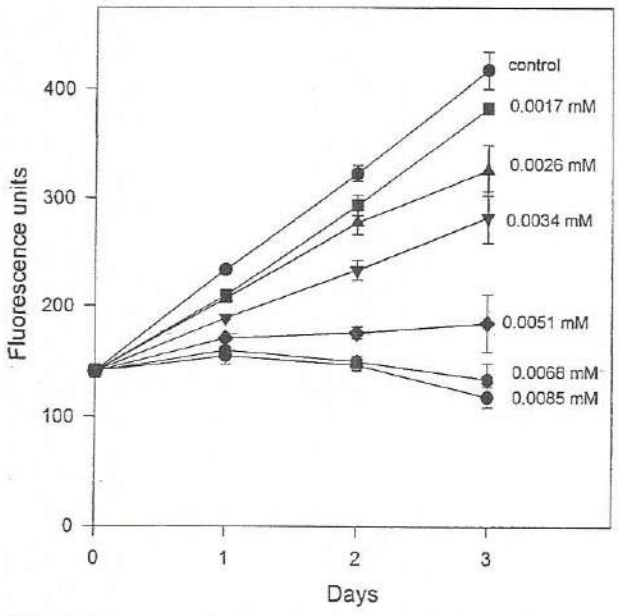


Fig. 3. Time course of S-G cell growth to a continuous 3–day exposure to sanguinarine chloride. Cellular DNA was estimated with the fluorochrome, Hoechst 33258, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Statistically significant (P≤0.05 or less) differences between control and experimental initially occurred at 0.0051 mM sanguinarine chloride on day 1 and at 0.0034 mM on days 2 and 3.

Results

Concentration-response cytotoxicity curves, as quantitated with the neutral red (NR) assay, for a 24-hr exposure of the S-G epithelial cells, HGF-1 fibroblasts, KB carcinoma cells, and PGC explant cells are shown in fig. 1. Initial toxicity (P≤0.05 or less) occurred at 3.4 μM sanguinarine chloride for the PGC cells, 5.1 μM for the S-G and KB cells, and 8.5 μM for the HGF-1 cells. Their respective NR_{50} values (i.e., the concentration of sanguinarine chloride that resulted in a 50% decrease in NR uptake relative to untreated controls), in μM, were 3.9±0.34 (PGC), 6.4±0.50 (KB), 7.6±0.45 (S-G), and 10.9±0.47 (HGF-1).

The morphology of the S-G cells grown in control medium is shown in fig. 2A. When freshly seeded, the S-G cells are pleomorphic, exhibiting both elongated and polygonal shapes. Later, they form a continuous sheet of polygonal cells. In the presence of sanguinarine chloride (fig. 2B), the cells exhibit vacuolization and some multinucleation. The HGF-1 fibroblasts occur as spindle shaped cells in normal medium (fig. 2C) but in the presence of sanguinarine they form enlarged, flattened cells, which subsequently, with increasing concentration, contract, giving rise to grotesque, irregular formations (fig. 2D). The explant cells (PGC) consisted both of epithelial cells and fibroblasts (fig. 2E); in the presence of sanguinarine, vacuolization and cell enlarge-

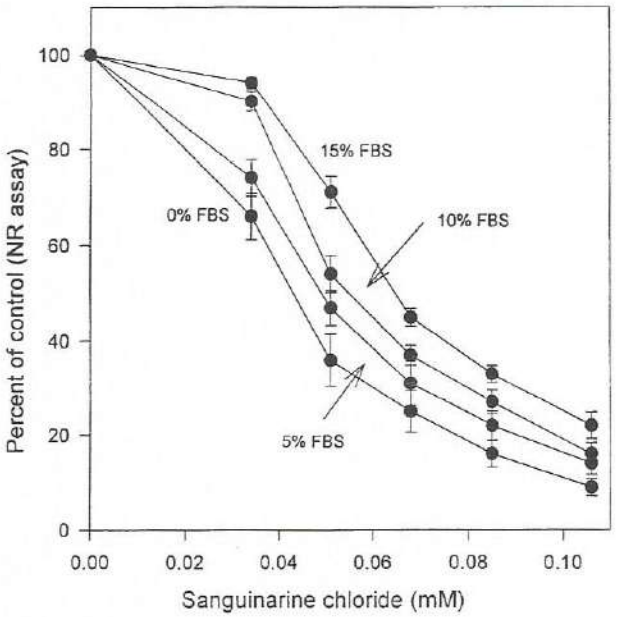


Fig. 5. Effect of foetal bovine serum (FBS) on a 1-hr exposure of sanguinarine chloride to S-G cells. Survival was quantitated with the neutral red (NR) assay. Mean percentages of control were computed by comparisons of the NR accumulated by the treated cells to that accumulated by the control cells. Statistically significant ($P \leq 0.01$) protection of sanguinarine chloride toxicity occurred only at 15% FBS.

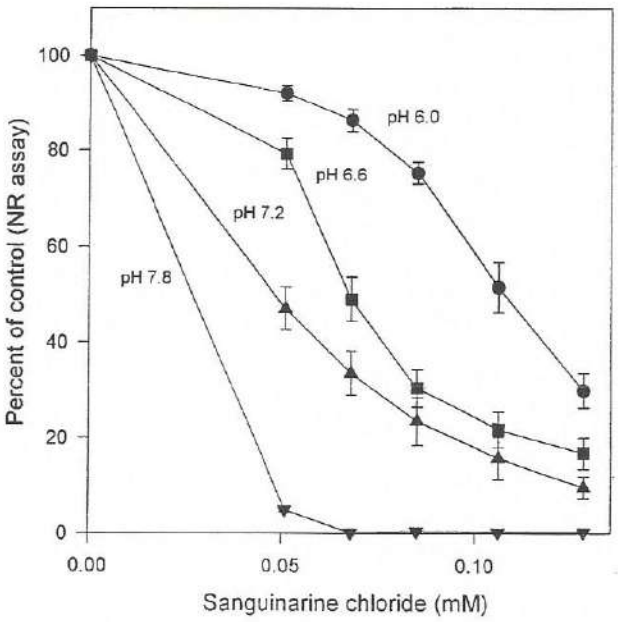


Fig. 6. Effect of pH on a 1-hr exposure of sanguinarine chloride to S-G cells. Survival was quantitated with the neutral red (NR) assay. Mean percentages of control were computed by comparisons of the NR accumulated by the treated cells to that accumulated by the control cells. Toxicities at all pH values by concentrations of 0.051 and 0.068 mM sanguinarine chloride were statistically different ($P \leq 0.05$ or less) from each other

ment were noted (fig. 2F). The KB cells, both in control (Fig. 2G) and in experimental (fig. 2H) medium are piling, characteristic of tumor cells; vacuolization was noted in the presence of sanguinarine chloride.

Further studies focused on the S-G human gingival epithelial cell line, which has previously been tested with other dental materials (Kasten *et al.* 1989 & 1990; Rawls *et al.* 1990; Babich *et al.* 1995; Sinensky *et al.* 1995).

The effect of sanguinarine chloride on proliferation of the S-G cells exposed for 3 days is shown in fig. 3. A rapid linear increase in growth was noted for the control, with a doubling time of 2 days. Although linearity was observed at 1.7 to 3.4 μM sanguinarine chloride, a lag in growth kinetics was also apparent. Essentially no growth was observed after a 3 day exposure to sanguinarine chloride at 5.1 μM and greater.

Table 1.

Cytotoxicity of sanguinarine chloride to S-G gingival epithelial cells, as mediated by an S9 hepatic microsomal fraction.

	Survival, as percent of control[a]		
Sanguinarine chloride	no S9 +NADP	S9 −NADP	S9 +NADP
0.007 mM	16±3.1	7±2.2	69±10.8[b]
0.008 mM	14±2.5	5±1.4	54±7.0[b]

[a] Survival was quantitated with the neutral red (NR) assay after a 1-day exposure.

[b] Significant at $P \leq 0.01$ when compared to the exposure conditions of no S9+NADP and of S9−NADP.

The effect of sanguinarine chloride on the integrity of the plasma membrane was assessed by the release of LDH from S-G cells during a 3 hr exposure. Significant ($P \leq 0.01$) release of LDH was initially detected at 0.1275 mM sanguinarine chloride (fig. 4).

Fig. 5 shows the toxicity of sanguinarine chloride to S-G cells in the absence and presence of FBS. Although there was a trend of a progressive reduction in the cytotoxicity of sanguinarine chloride with increasing concentration of FBS, significant differences ($P \leq 0.01$) between control (sanguinarine, no FBS) and experimental (comparable concentration of sanguinarine, with FBS) were evident only at 15% FBS.

Increasing the pH, increased the toxicity of a 1 hr exposure to sanguinarine chloride (fig. 6). Thus, for example, at 0.051 and 0.068 mM sanguinarine chloride, significant differences ($P \leq 0.05$ or less) in toxicity were noted among all the pH values tested. The NR_{50} values, in mM, for a 1 hr exposure to sanguinarine chloride in PBS adjusted to the varied pH values, in mM, were 0.108±0.0039 at pH 6.0, 0.073±0.0037 at pH 6.6, and 0.043±0.0081 at pH 7.2. At pH 7.8, the NR_{50} value was estimated at 0.025 mM.

The toxicity of sanguinarine chloride was evaluated in the absence and presence of an Aroclor-induced rat S9 hepatic microsomal fraction. This system requires the addition of exogenous NADP for activation of the enzymes. As the liver is the main organ for the metabolism of xenobiotics, mammalian microsomal fractions (such as the 9,000×g (S9) fraction of liver from rats treated with inducers of mixed-

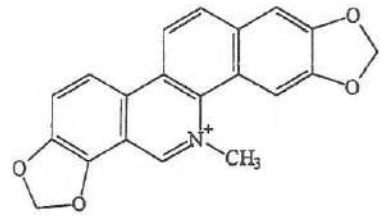
Table 2.

Cytotoxicity of dental products to human cell lines in culture.

Cell type	24-Hr NR_{50} (mM) value[a]	
	Sanguinarine chloride	Chlorhexidine digluconate[b]
S-G	0.0076	0.011
KB	0.0064	0.011
HGF-1	0.0109	0.017

[a] The concentration of test agent that reduced the uptake of neutral red (NR) by 50% of control cultures after a 1-day exposure.
[b] Data from Babich *et al.* (1995).

function oxidase activity) have been employed as the xenobiotic activating system in *in vitro* cytotoxicity assays (Babich *et al.* 1987; Borenfreund & Puerner 1987). Since studies with FBS showed that the toxicity of sanguinarine chloride was modified in the presence of protein, an internal protein control (S9 microsomal fraction without the addition of NADP) was incorporated into this study to examine a possible adsorption of the test agent to the proteins of the microsomal mixture. As noted in table 1, the toxicity of sanguinarine chloride to the S-G cells was significantly ($P \leq 0.01$) lessened only in the presence of the hepatic S9 microsomal fraction activated by NADP.



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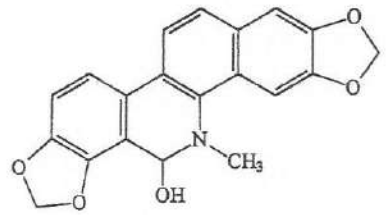

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Fig. 7. The iminium (structure I) and alkanolamine (structure II) form of sanguinarine chloride.

Discussion

As expected, the primary cell culture was the most sensitive to sanguinarine chloride, since these cells had to adapt to culture conditions. Among the established cell lines, sanguinarine chloride exhibited approximately equivalent cytotoxicity to the S-G epithelial cells and the KB carcinoma cells, whereas the HGF-1 fibroblasts were more resistant. This was similar to the response of these cell lines to chlorhexidine digluconate, used to prevent dental plaque and gingivitis. For each cell line, sanguinarine chloride was more potent than chlorhexidine digluconate (table 2).

The relatively high concentrations of sanguinarine chloride needed to cause leakage of LDH from S-G cells, as compared to levels needed to cause other cytotoxic effects, suggests that its prime mode of toxicity is not at the level of the plasma membrane. This is in contrast to chlorhexidine digluconate which preferentially damages the plasma membrane (Helgeland *et al.* 1971; Babich *et al.* 1995).

As assessed by DNA quantitation, S-G cells maintained continuously in medium amended with sanguinarine chloride at 1.7 to 3.4 μM exhibited a prolonged lag in their growth. Such a subtle adverse effect of cell replication may reflect the ability of sanguinarine chloride to intercalate DNA (Sen & Maiti 1994) and/or to interfere with tubulin assembly (Wolff & Knipling 1993). Such interference with formation of spindle fibers may be a contributing factor to endoreduplication noted in our studies with the sanguinarine-treated S-G cells.

In the studies reported herein, metabolic activation by an S9 hepatic microsomal fraction greatly lessened the cytotoxic potency of sanguinarine chloride. There are, as yet, no data in the literature on the identification of specific metabolic products of sanguinarine (Becci *et al.* 1987; Frankos *et al.* 1990).

The lessening of the potency of sanguinarine by FBS was, apparently, due to the sorption of the test agent to the sera, thereby reducing the bioavailability of sanguinarine chloride to the cells. This is in accord with Paulova & Slavik (1993) who showed binding of sanguinarine chloride to human serum albumin.

Sanguinarine exhibits pH dependent structural equilibrium between the iminium form (structure I) and the alkanolamine form (structure II) with a pK value of 7.4 (fig. 7) (Maiti *et al.* 1983). Both forms are quite stable (Jones *et al.* 1986). The effect of pH on sanguinarine toxicity is in accord with the conversion of the cationic iminium form to the alkanolamine form. This conversion from the iminium ion to the alkanolamine form enhances the potency of sanguinarine chloride, probably by increasing the cellular availability of the alkanolamine form due to its greater lipophilicity. However, once internalized into the cytoplasm, the pH of that latter environment may favor the reconversion to the iminium ion, which may be the chemical form that evokes a cytotoxic response. The potency of sanguinarine has been correlated with the reaction of its iminium cationic form with nucleophiles. For example, sanguinarine forms reversible adducts with Na^+/K^+ ATPase and tubulin through nucleophilic attack by the proteins (probably, sulfhydryl (-SH) groups) on the iminium group (Cohen *et al.* 1993; Wolff & Knipling 1993). Furthermore, by intercalation, the iminium form of sanguinarine interacts with DNA (Sen & Maiti 1994).

Acknowledgements

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