THE EFFECT OF NICOTINE ON REPRODUCTION AND ATTACHMENT OF HUMAN GINGIVAL FIBROBLASTS *in vitro*

by

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This thesis submitted by Mark Eugene Peacock, D.M.D. has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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INTRODUCTION

Statement of the Problem

The purpose of this research was to determine the following: (1) whether nicotine has any effect on the growth and metabolism of human gingival fibroblasts, and (2) whether the ability of human gingival fibroblasts to attach to a substrate is affected by exposure to nicotine.

Literature Review

The role of tobacco use in the etiology of periodontal disease is not clear; however, many practitioners believe there is a strong positive correlation between the use of tobacco and increased incidence and severity of periodontal disease (1-3). Increased amounts of plaque and debris in smokers have been found in some studies (4-6), while others reported a decrease in plaque accumulation (7-9). Smokers may have greater amounts of calculus present (2,6,7,9) and a higher incidence of staining, which can act as a nidus for subsequent calculus deposition (10). Gingivitis also appears to be more prevalent in tobacco users (2,7,11), although some investigators question the degree of gingival inflammation present in smokers as compared to non-smokers (8,9). Preber and Kant, in a study of 15-year-old school children, found no significant differences in gingival inflammation between nonsmokers and smokers when subjects with the same

level of oral hygiene were matched (12). Others have reported higher incidences of acute necrotizing ulcerative gingivitis in smokers, where the smoke or its components seem to potentiate the effects of stress and oral sepsis (13,14).

Similarly, wound healing is perceived by some to be influenced by the use of tobacco products. Some clinicians have experienced less success with certain surgical procedures, such as free gingival grafts, in smokers (15). Preber and Bergström showed a lesser response in reduction of the periodontal probing depth following non-surgical therapy in smokers when compared to non-smokers (16). Smoking also impaired the healing response after periodontal surgery when compared to post-surgical healing in non-smokers (17). Decreased healing associated with tobacco use also has been reported after oral surgical procedures (18), plastic surgery procedures for the removal of wrinkles (19), head and neck skin grafting (20), and duodenal ulcer treatment (21). However, the actual nature of the relationship between cigarette smoke and its components and the response to treatment seen in periodontal therapy still remains unclear (22).

Cigarette smoke contains a complex mixture of substances including nicotine, a variety of nitrosamines, trace elements, and a variety of poorly characterized substances. Cellular responses to these substances vary widely and may relate to specific components of the smoke, the amount of such components, and the cell type. For example, the ability of oral polymorphonuclear leukocytes (PMNs) to survive in the presence of smoke is decreased, and their phagocytotic activity and chemotactic ability are also adversely affected (23-25). Kraal and Kenney reported that there was no difference in PMN migration in smokers versus non-smokers when the smokers refrained from smoking

overnight (26). In addition, degranulation of mast cells was stimulated (27), lymphocyte viability decreased (27), lymphocyte proliferation suppressed (28), and antibody production reduced in the presence of cigarette smoke (28). Lindemann and Park showed that one of the major components of fine-cut smokeless tobacco, benzo(a)pyrene, suppressed DNA synthesis of lymphokine-activated killer cells (LAK), as well as suppressing LAK cytotoxicity against tumor targets (29). Some studies have shown that nicotine suppressed DNA synthesis in lymphocytes and HeLa cells (30,31). Another study suggested that the DNA synthesis rates of murine peritoneal macrophages and murine embryonic fibroblasts were elevated when exposed to fresh cigarette smoke (32). Galvin et al have reported that nicotine and smokeless tobacco extract both activated glycolysis and suppressed bone collagen synthesis and mitochondrial activity in chick embryo tibial bone explant preparations (33). In another study these same individuals showed that smokeless tobacco extract inhibited collagen synthesis in osteoblasts. There was no effect on lactate production (34). Lenz et al demonstrated that nicotine inhibits collagen synthesis and alkaline phosphatase activity and stimulates DNA synthesis in osteoblast-like cells isolated from chick calvariae (35). A more recent study concluded that nicotine inhibiteed cellular growth and stimulated alkaline phosphatase activity in rat osteoblast-like cells (36).

Nicotine also has been shown to increase the production of type III collagen by human diploid fibroblasts in culture by one-half, while at the same time hindering secretion of collagen (37). Litwin et al suggest that embryonic fibroblast transformation is mostly unaffected by tobacco smoke condensate fractions of 10 μg/ml or lower (38).

Recent experiments have shown that nicotine non-specifically binds to gingival fibroblasts and is taken up by the fibroblasts, resulting in high intracellular levels; subsequently, a portion of the nicotine is rereleased into the environment both as unmetabolized nicotine, as well as its metabolic products (39). Other studies utilizing human foreskin fibroblasts revealed that nicotine, while not preventing the attachment of the cells, may alter their orientation and nature of attachment to glass and tooth root surfaces (40).

The effects of nicotine and cigarette smoke on the oral mucosal vasculature has also been the focus of several studies. Clarke et al discovered that when nicotine was administered systemically to rabbits, an initial vasodilation followed by a rapid vasoconstriction occurred (41,42). The reduced gingival blood flow due to vasoconstriction one may experience when exposed to cigarette smoke is supported in a study by Bergstrom et al on smoking and experimental gingivitis (43). Conversely, others have shown an increase in gingival blood flow in humans when exposed to cigarette smoke (44). A recent experiment by Johnson et al involved the systemic administration of nicotine to Sprague-Dawley rats via subcutaneous minipumps over a two-week period. After the animals were sacrificed, biopsies were taken from the palate, maxillary gingiva, and buccal mucosa. Tissue sections were then incubated and subsequently measured for alkaline phosphatase, a capillary marker. It was demonstrated that the total capillary fragment length and capillary height in the nicotine-treated group was significantly less than that of the control group (45).

Numerous inquiries into the relationship between smoking and alveolar bone height have been made. Daniell attributed increased alveolar bone loss to tobacco

smoking (46), and similar findings were revealed by Christen et al in smokeless tobacco users (47). Bergström et al suggest that an increased loss of periodontal bone height is significantly related to smoking (48). While other investigations have come to a similar conclusion, i.e. increased bone loss in smokers (2,4,9,11,49-54), Preber et al, using Swedish army conscripts, reported the same degree of alveolar bone loss in smokers when compared to non-smokers (55). Studies by Bergström and Eliasson have shown greater bone loss in subjects that smoked than non-smokers, irrespective of the amount of plaque present (56,57). Even the bone mineral content, particularly of the periodontal hard tissues, has been reported to be decreased in smokers compared to non-smokers (58,59). While some studies suggest a direct influence of tobacco and/or its metabolites on the health of the alveolar bone, this has yet to be confirmed.

Several bacteriological investigations have attempted to demonstrate significant differences in the proportion of aerobic to anaerobic bacteria in smokers and non-smokers. Kenney et al tested the hypothesis that cigarette smoke could cause a lowered oxidation-reduction potential (Eh) in the oral tissues, and that this could subsequently cause an increase in the anaerobic flora (60). They found a drop in Eh values with an increase in the pH of the tissues analyzed, although no differences in the proportion of aerobes to anaerobes were found. Bastiaan and Waite studied the effects of tobacco smoke on plaque development and discovered a statistically significant increase in the number of Gram-positive bacteria in smokers during the first few days of plaque formation (8). In a study comparing smokers to non-smokers, Colman et al found fewer Gram-negative aerobes (*Neisseria*) in the oral flora of smokers (61). The results of this study parallels

the findings of subsequent investigators in which Gram-negative bacteria (*Branhamella catarrhalis*, *Neisseria perflava* and *Neisseria sicca*) were more sensitive to the effects of cigarette smoke than certain species of Gram-positive bacteria (*Streptococcus mitis*, *Streptococcus salivarius* and *Streptococcus sanguis*) (62,63). While it appears from these studies that shifts in the proportion of certain bacteria are possible when exposed to tobacco smoke, whether these changes play a significant role in the periodontal disease process remains to be substantiated.

The consequences of tobacco use and smoking can adversely affect a variety of other tissues and cell types. These include an increased risk of cancer, cardiovascular disease, chronic obstructive pulmonary disease, and increased hazards to pregnant women and the fetus (64-68). Most studies on smoking, excluding those on carcinogenesis, have been directed at the factors that cause cardiovascular disease, primarily arteriosclerotic disease (69). Angina pectoris is made worse by nicotine in many patients and may contribute to an increased incidence of myocardial infarction and sudden death from coronary artery disease in smokers (70). Animal studies have shown that exposure to high levels of carbon monoxide increased atherogenesis in animals fed high cholesterol diets, but not in animals fed regular diets. Nicotine ingestion associated with cigarette smoking has been shown to cause an increase in plasma free fatty acids, indirectly caused by endogenous catecholamine release (71,72). Although the apparent increased risk to ischemic heart disease cannot be explained by the pharmacological action of nicotine alone, nicotine may act as an additional etiological factor in diseases of the cardiovascular system (69).

The adrenergic stimulation of endogenous catecholamines produced by nicotine administration can increase platelet adhesiveness and aggregation, thereby accelerating thrombus formation in blood vessels (70). One may conclude that these factors might possibly contribute to vascular obstruction and an increased incidence of embolism in individuals with advanced atherosis.

There have been several studies that have attempted to verify a negative correlation between Parkinson's disease and smoking (73-75). It is known that nicotine acts as a stimulus to bring about the circulation of dopamine. Drug therapy for Parkinson's disease is aimed at correcting or modifying neurotransmitter defects by either inhibiting the effects of acetylcholine or enhancing the effects of dopamine (76). Whether smoking or other tobacco use has a role in the development of Parkinson's disease remains to be established.

The effects of smoking and nicotine on the development of ulcerations of the gastrointestinal tract have been the focus of investigation in animals and humans (77,78). Hydrogen ion secretion, gastric pepsin output and mucous production does not appear to be stimulated by either smoking or nicotine (69). However, the following pathophysiological effects that may be associated with ulcer formation have been found: smoking and nicotine inhibit the secretion of bicarbonate from the pancreas; duodenal bile reflux is increased by smoking, possibly allowing the stomach mucosa to become more susceptible to attack by pepsins 1 and 3; and, pepsin 1, which is a more active degrader of collagen than pepsin 3, appears to be elevated in smokers (69,77). Although studies have failed to explain how nicotine and smoking promote ulcer formation, there appears

to be a connection between smoking and peptic ulcer disease, possibly by alterations in cellular metabolism or behavior.

In women smoking during pregnancy, exposure of the developing fetus to nicotine and its metabolites can cause significant problems when compared with non-smokers (79,80). There is evidence to suggest that tobacco use by pregnant women may lead to a higher number of spontaneous abortions and an increased infant death rate (79,81). It has been demonstrated that smoking has very little effect on the secretion of milk by the mother, even though nicotine is accumulated to some extent in breast milk (82). Further human studies are needed on smoking during pregnancy in order to elucidate any possible detrimental effects to the developing fetus.

Specific Aims

The specific aims of this study were as follows:

1. To determine the effect of nicotine on human gingival fibroblast reproduction after short- and long-term exposure.
2. To ascertain whether any effect(s) seen upon human gingival fibroblast growth persist after removal of nicotine.
3. To determine if nicotine has any impact upon fibroblast attachment.

The ability of fibroblasts to reproduce and attach to substrates is of paramount importance in re-establishing the lost connective tissue attachment after periodontal therapy. Since tobacco components have been thought to affect the reattachment response, understanding how the cells behave in the presence of these components will

provide valuable information concerning the perceived decreased response to therapy seen in smokers.

MATERIALS AND METHODS

Materials

Gingival Fibroblast Cultivation:

Human gingival fibroblasts (HGF) were obtained as primary cultures from the Medical College of Georgia (MCG) School of Dentistry, Department of Oral Biology. The HGF cultures were procured from gingival biopsies taken during routine periodontal surgical procedures on adult patients. Cultures of gingival fibroblasts were subcultured in 75 cm^2 tissue culture flasks and maintained in Eagle's Minimum Essential Medium (EMEM) with Earle's Salts and glutamine. The EMEM was supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). The cells were incubated at 37°C in an atmosphere of 95% air/5% CO_2. The pH of the tissue culture medium was adjusted to 7.4 with 1 N HCl and/or 1 N NaOH.

Nicotine Standard Preparation:

A 1-mg/ml stock solution of nicotine obtained from ICN Biomedicals was prepared by pipetting 100 µl of nicotine (the density of nicotine is 1.0094 g/ml, so 100 µl weighs approximately 100 mg) and 100 µl of dimethylsulfoxide (to assist in dissolution) into a 100-ml volumetric flask and filling to the mark with ultrapure water. The resulting solution was 100 mg/100 ml or 1 mg/ml nicotine, and 0.1 ml/100 ml (v/v)

or 0.1% dimethylsulfoxide. The stock standard was stored at 4°C for no longer than two weeks.

To prepare the working nicotine solution, 100 ml of medium was pipetted into a sterile tissue culture flask. 415 µl of medium was removed and replaced with 415 µl of stock 1-mg/ml nicotine solution. The solution was mixed well on a gyratory mixer. The resulting solution was 25.6 µM nicotine in medium. Working solutions were made fresh for every experiment and disposed of after the experiment.

Methods

Cell Splitting and Counting:

Confluent cultures (passage 5-10) of HGF were split by decanting the medium from the tissue culture flask, rinsing the cell layer and flask with approximately 30 ml of sterile phosphate- buffered saline (PBS), and adding 2.5 ml of 0.5% trypsin/0.1% EDTA. The trypsin was layered evenly over the cell layer and incubated at 37°C for 5 minutes, or until the cells began to pull away from the flask. The trypsin was gently decanted and 30 ml of tissue culture medium added. The cells were dislodged from the flask then suspended by mixing on a gyratory mixer.

If the cells were to be passed, another 60 ml of medium was added to the flask, and 30-ml aliquots added to two new flasks, leaving 30 ml in the original flask. This yielded a 1:3 split of the original cells.

If the cells were to be used in an experiment, 100 µl of cell suspension was added to 300 µl of 0.5% trypan blue (1:4 dilution) in a 12 x 75 mm test tube. The cells were

mixed well and allowed to sit for 5 to 15 minutes to allow the cells to take up the trypan blue. The non-stained (viable) cells were then counted in a Neubauer hemocytometer on an Olympus BH2 microscope.

Colorimetric Assay Procedure:

The assay used to determine cell numbers involved 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT), which is a reflection of mitochondrial dehydrogenase activity in living cells (83). The tetrazolium ring in MTT is cleaved by active mitochondria to yield a dark blue formazan product which can be easily read on a scanning multiwell spectrophotometer (ELISA reader). Slater et al in 1963 performed the studies which determined that tetrazolium salts measured the activity of several dehydrogenase enzymes, particularly succinic dehydrogenase (84). The assay includes the addition of a sterile MTT dye solution to culture wells containing the experimental substance to be measured. Cells are incubated for an additional 4 hours, during which time they convert the tetrazolium substrate of the dye solution into a blue formazan product that is insoluble in culture medium. A Solubilization Solution is then added to dissolve the formazan product and produce a colored solution suitable for absorbance measurement. Absorbance is directly proportional to the number of living cells present.

Effect of Nicotine on HGF Attachment:

A working solution of 25.6-μM nicotine was prepared from the stock as described above, and 100 μl of medium was pipetted into columns 1-10 of six 96-well tissue culture

plates. 100 µl of 25.6 µM nicotine was pipetted into column 11 of each plate. Another 100 µl of 25.6 µM nicotine was pipetted into columns 10. The resulting 12.8 µM solution was mixed well by pipetting up and down with the pipettor twice, then 100 µl was transferred to columns 9. This serial dilution was continued down to columns 2. The addition of 100 µl of cell suspension (1×10^5 cells/ml) to columns 1-6 yielded 1×10^4 cells/well and the following concentrations of nicotine:

1	2	3	4	5	6
Control (0 µM)	0.025 µM	0.05 µM	0.1 µM	0.2 µM	0.4 µM

The average physiological concentration for a smoker has been previously determined to be approximately 0.1 µM for nicotine (85-87). The plates were incubated at 37°C in a 5% CO_2 atmosphere for 10, 20, 30, 45 or 60 minutes. At the end of the incubation, the plates were emptied by inversion and blotting onto plastic-backed absorbent paper. The wells were washed twice with 200 µl of sterile PBS and emptied and blotted as before to remove any unattached cells. 100 µl of fresh medium and 15 µl of sterile MTT dye solution were added to each well. The plates were incubated for 4 hours at 37°C in 5% CO_2 to allow the cells to convert the yellow MTT to the insoluble blue formazan crystalline product. At the end of the incubation period, 100 µl of Solubilization Solution was added to each well. The plates were sealed by sandwiching a plate-sized piece of Parafilm between the plate and the cover and incubated overnight at 37°C to solubilize

the formazan product. The next morning the plates were read in the microplate reader at 570 nm.

Effect of Nicotine on HGF Mitochondrial Enzyme Activity:

Six 96-well tissue culture plates containing 1 x 10^4 cells/well were incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. Serial dilutions of a 25.6-µM nicotine working standard were made in tissue culture flasks with medium to produce concentrations of 0.025, 0.05, 0.1, 0.2 and 0.4 µM. The plates were emptied by inversion and blotting onto plastic-backed absorbent paper and then washed once with 200 µl of sterile PBS. 200 µl of medium was pipetted into column 1 of each plate. 200 µl of nicotine solution was pipetted into columns 2-6 of each plate to yield the following concentrations:

1	2	3	4	5	6
Control (0 µM)	0.025 µM	0.05 µM	0.1 µM	0.2 µM	0.4 µM

The plates were incubated at 37°C in 5% CO_2 for 0, 10, 20, 30, 45 or 60 minutes. Following incubation, the plates were emptied by inversion and blotting onto plastic-backed absorbent paper. The wells were washed twice with 200 µl of sterile PBS, and then 100 µl of fresh medium and 15 µl of MTT dye solution were added to each well. The plates were incubated at 37°C and the color intensity determined as described above.

Effect of Continuous Exposure of Nicotine on HGF Cell Growth:

Four 96-well tissue culture plates containing 1 x 10^4 cells/well were incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. Serial dilutions of a 25.6 µM nicotine working standard were made with medium to produce nicotine solutions of 0.025, 0.05, 0.1, 0.2 and 0.4 µM. The plates were emptied by inversion and blotting onto plastic-backed absorbent paper and then washed once with 200 µl of sterile PBS. 200 µl of medium was pipetted into column 1 of each plate. 200 µl of nicotine solution was pipetted into columns 2-6 of each plate to yield the following concentrations:

1	2	3	4	5	6
Control (0 µM)	0.025 µM	0.05 µM	0.1 µM	0.2 µM	0.4 µM

The plates were incubated at 37°C in 5% CO_2 for 4, 20, 24 or 48 hours. Following incubation, the plates were emptied by inversion and blotting onto plastic-backed absorbent paper. The wells were washed twice with 200 µl of sterile PBS, and then assayed by the MTT procedure as described above.

Effect of a 1-Hour Pre-exposure of Nicotine on HGF Cell Growth:

Five 96-well tissue culture plates containing 1 x 10^4 cells/well were incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. Serial dilutions of a 25.6 µM nicotine working standard were made with medium to produce nicotine solutions of 0.025, 0.05, 0.1, 0.2 and 0.4 µM. The plates were emptied by inversion and

blotting onto plastic-backed absorbent paper and then washed once with 200 µl of sterile PBS. 200 µl of medium was pipetted into column 1 of each plate. 200 µl of nicotine solution was pipetted into columns 2-6 of each plate to yield the following concentrations:

1	2	3	4	5	6
Control (0 µM)	0.025 µM	0.05 µM	0.1 µM	0.2 µM	0.4 µM

The plates were incubated at 37°C in 5% CO_2 for 1 hour. Following incubation, the plates were emptied by inversion and blotting onto plastic-backed absorbent paper. The wells were washed twice with 200 µl of sterile PBS. 200 µL of fresh medium was added to each well, and the plates were incubated at 37°C in 5% CO_2 for 0, 1, 20, 24 or 48 hours. Following this incubation, the plates were emptied by inversion and blotting and washed twice with 200 µl of sterile PBS, and then assayed by the MTT procedure as described above.

Effect of a 6-Hour Pre-incubation of Nicotine on HGF Cell Growth:

Five 96-well tissue culture plates containing 1 x 10^4 cells/well were incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. Serial dilutions of a 25.6 µM nicotine working standard were made with medium to produce nicotine solutions of 0.025, 0.05, 0.1, 0.2 and 0.4 µM. The plates were emptied by inversion and blotting onto plastic-backed absorbent paper and then washed once with 200 µl of sterile

PBS. 200 µl of medium was pipetted into column 1 of each plate. 200 µl of nicotine solution was pipetted into columns 2-6 of each plate to yield the following concentrations:

1	2	3	4	5	6
Control (0 µM)	0.025 µM	0.05 µM	0.1 µM	0.2 µM	0.4 µM

The plates were incubated at 37°C in 5% CO_2 for 6 hours. Following incubation, the plates were emptied by inversion and blotting onto plastic-backed absorbent paper. The wells were washed twice with 200 µl of sterile PBS. 200 µl of fresh medium was added to each well, and the plates were incubated at 37°C in 5% CO_2 for 0, 1, 20, 24 or 48 hours. Following this incubation, the plates were emptied by inversion and blotting and washed twice with 200 µl of sterile PBS, and then assayed by the MTT procedure as described above.

Analysis of Statistics:

The data from each investigation were statistically evaluated using a univariate analysis by the one-way analysis of variance with replicates. In addition, the analysis employed Fisher's LSD test for each of the time periods.

RESULTS

Standardization of the MTT Assay:

To determine whether the MTT assay reflected cell numbers a standard curve comparing absorbance and cell numbers was prepared by plating fixed numbers of cells, then determining the absorbance at 570 nm. Figure 1 shows a linear relationship between cell number and absorbance in the range of 438 to 14,000 cells. The effective limit of this procedure is approximately 1 x 10^5 cells/well.

Mitochondrial Enzyme Activity:

To ascertain that the MTT assay reflected cell numbers and not altered enzyme activity, cells were plated and allowed to attach to the surface of the dish. They were then exposed to varying concentrations of nicotine in the range of 0-0.4 μM for up to 4 hours, and the dehydrogenase activity determined by the MTT assay. Upon exposure to nicotine, dehydrogenase activity decreased over time for each concentration of nicotine, reaching its nadir at 45 minutes; however, by 4 hours enzyme activity had recovered to approximately 100% of control values (Figure 2). As the concentration of nicotine increased from 0.025 μM to 0.4 μM, the level of enzymatic activity decreased comparably, and this was epecially evident at the 45-minute time point. Therefore, the lowest level of enzyme activity occurred at 45 minutes with the 0.4 μM nicotine

concentration when compared to the control, and this difference was significant, while at this same time period, the 0.025 μM nicotine concentration was only mildly affected and the decrease was not statistically significant. The rates of recovery for 0.025-0.2 μM were very similar. The rate of recovery for 0.4 μM was greater so that all cells had recovered to control levels by 4 hours, further emphasizing the transient nature of the depression of enzyme activity. The statistical evaluations of the degree of change versus time and nicotine concentration are shown in Table I. Results further emphasize the transient time/dose relationship between nicotine concentration and dehydrogenase activity.

HGF Attachment in the Presence of Nicotine:

Cells began to attach to the tissue culture plate surfaces very soon after being plated, whether in the presence or absence of nicotine. The number of cells attached was determined by the MTT assay at 10, 20, 30, 45 and 60 minutes after inoculation into plates in the presence of varying concentrations of nicotine. The number of fibroblasts that attached upon exposure to the various concentrations of nicotine generally increased over time and with increasing nicotine concentrations (Figure 3). For cells exposed to 0.4 μM nicotine, the number attached had nearly doubled after 1 hour as compared to the control cells. At concentrations of nicotine $\geq$ 0.1 μM the cell numbers were significantly greater than control cultures at all time periods ($p < 0.05$), and by 60 minutes, the numbers at all nicotine concentrations were significantly greater than controls ($p < 0.05$) (Table II).

Photographs taken with phase contrast microscopy show that fibroblasts exposed to 0.4 μM nicotine tended to exhibit a more flattened appearance than the control cells, starting at the 30-minute time period (Figure 4). The margins appeared rough or crenated (somewhat suggestive of senescence). By 60 minutes the nicotine-exposed cells showed an even greater difference from control cells. The latter had a more raised appearance than the cells exposed to nicotine, which looked much flatter and appeared to be spread out over the culture plate surface (Figure 5). By 4 hours these differences disappeared and no morphologic differences were apparent between experimental and control cells.

Cell Reproduction Upon Continuous Nicotine Exposure:

Measurements of dehydrogenase activity showed that even though enzyme production was being inhibited initially, at the concentrations used, nicotine did not kill the cells. Indeed, by 4 hours, enzyme activity had returned to approximately 100% of control. Furthermore, as Figure 1 shows, absorbance is a reflection of the number of cells present. Therefore, MTT absorbance for time periods of 4 hours and longer, which equates with mitochondrial enzyme activity, reflects cell number. The lowest concentration of nicotine, 0.025 μM, produced a steady increase in fibroblast number over 48 hours and this was statistically significant at all times beyond 4 hours. The higher concentrations of nicotine generally resulted in decreased cell numbers after 20 hours compared to the levels at the start of the experiments, and this appeared to be concentration-dependent (Figure 6). However, by 24 hours, fibroblast numbers in cultures with nicotine began to increase although they were generally equal to control cultures.

Figure 1: *Standard curve for HGF using MTT assay. Absorbance is directly proportional to the number of cells.*

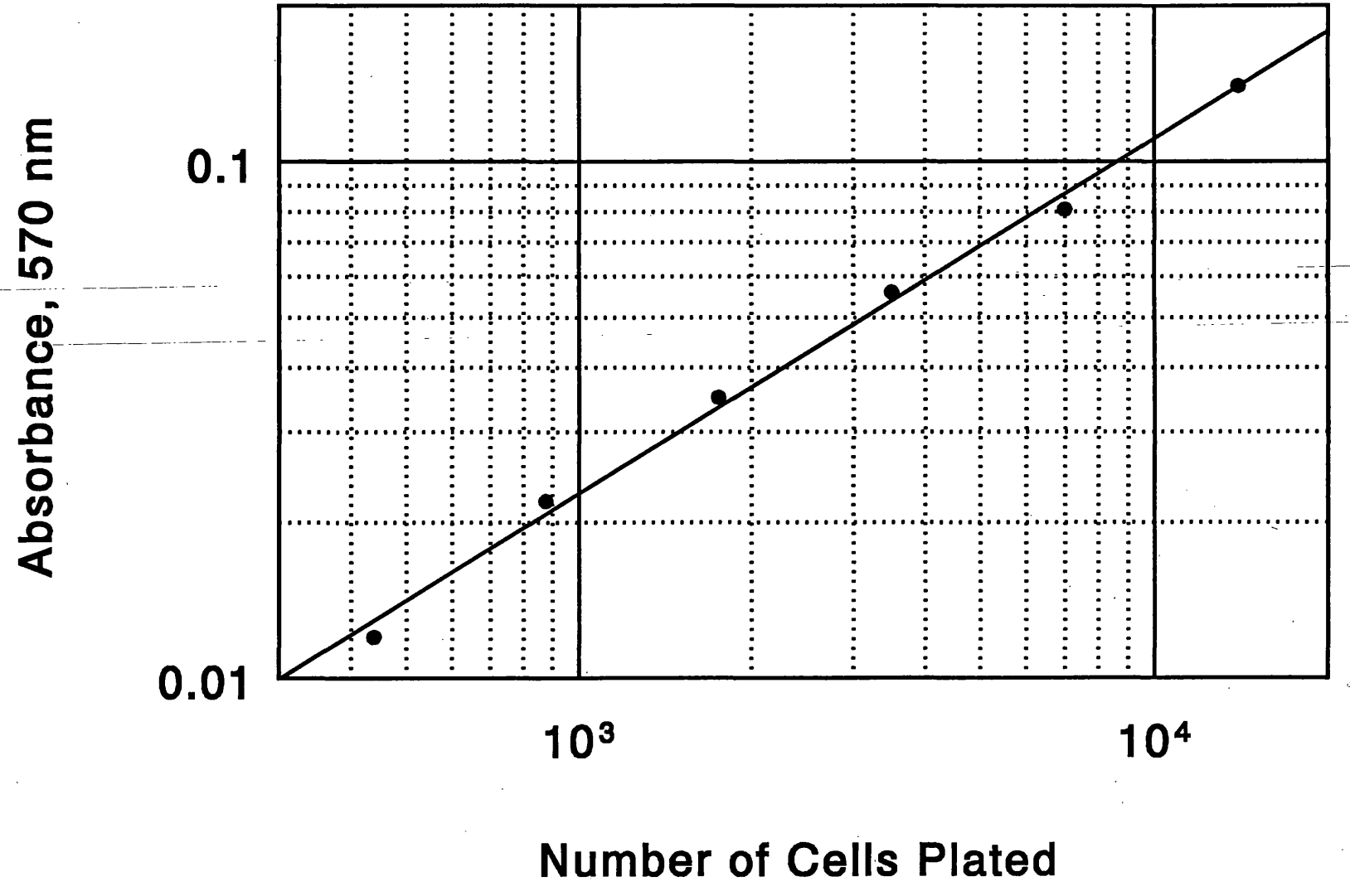

Absorbance, 570 nm
0.1
0.01
10^3
10^4
Number of Cells Plated

Figure 2: *Enzyme activity/growth of HGF cultures exposed to nicotine for up to 4 hours. Enzyme activity of cells exposed to 0.4 μM nicotine concentration was significantly different from control values for all time periods ($p < 0.05$). At the 30-minute time period, activity following exposure to all nicotine concentrations was significantly different from control levels ($p < 0.05$).*

- ● *0.025 μM nicotine*
- ○ *0.05 μM nicotine*
- ■ *0.1 μM nicotine*
- □ *0.2 μM nicotine*
- ▲ *0.4 μM nicotine*
- - - *Control*

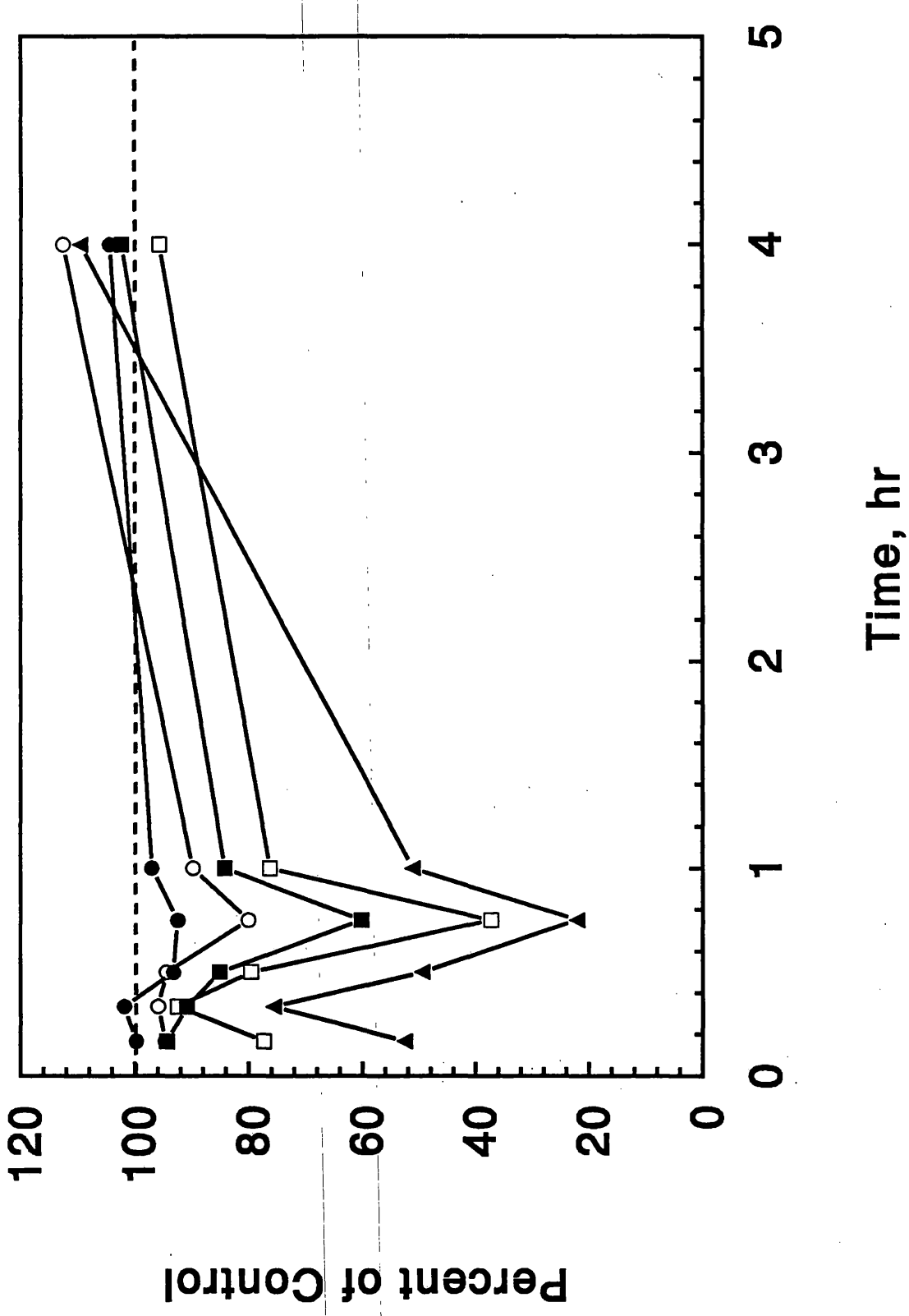

Percent of Control
120
100
80
60
40
20
0
0
1
2
3
4
5
Time, hr

Table I. *Mitochondrial Dehydrogenase Activity* at Varying Concentrations of Nicotine Over a 4-Hour Time Period*

	Nicotine Concentration (μM)						
Time	0	0.025	0.05	0.1	0.2	0.4	p**
10 min	100% (5.4%)	102.0% (5.3%) NS***	95.9% (3.5%) NS	96.6% (9.2%) NS	81.7% (11.1%) p<0.05	58.8% (9.0%) p<0.05	<0.0001
20 min	100% (4.1%)	99.6% (5.1%) NS	93.2% (2.6%) p<0.05	91.8% (6.0%) p<0.05	91.5% (2.0%) p<0.05	79.4% (3.4%) p<0.05	<0.0001
30 min	100% (7.4%)	94.2% (1.8%) p<0.05	93.5% (2.8%) p<0.05	85.7% (3.7%) p<0.05	80.7% (3.7%) p<0.05	54.3% (6.5%) p<0.05	<0.0001
45 min	100% (5.9%)	92.6% (5.7%) NS	80.7% (14.4%) p<0.05	59.3% (25.2%) p<0.05	38.1% (20.0%) p<0.05	20.3% (4.2%) p<0.05	<0.0001
60 min	100% (3.5%)	96.2% (5.2%) NS	87.7% (6.0%) p<0.05	83.4% (6.6%) p<0.05	77.6% (12.0%) p<0.05	53.0% (14.6%) p<0.05	<0.0001
240 min	100% (3.9%)	104.1% (4.3%) NS	110.4% (1.9%) p<0.05	102.3% (5.7%) NS	95.5% (3.7%) NS	109.3% (5.2%) p<0.05	<0.0001

*Percent of Control (0.00 μM) Activity (± S.D.)
**ANOVA
***Fisher's LSD Test (NS = Not Significant)

Figure 3: *Attachment of HGF to tissue culture plates when cells were exposed to nicotine. At all nicotine concentrations the numbers attached were significantly greater than those attached in control cultures ($p < 0.05$) at 45 minutes or longer.*

● *Control*
○ *0.025 μM nicotine*
■ *0.05 μM nicotine*
□ *0.1 μM nicotine*
♦ *0.2 μM nicotine*
◊ *0.4 μM nicotine*

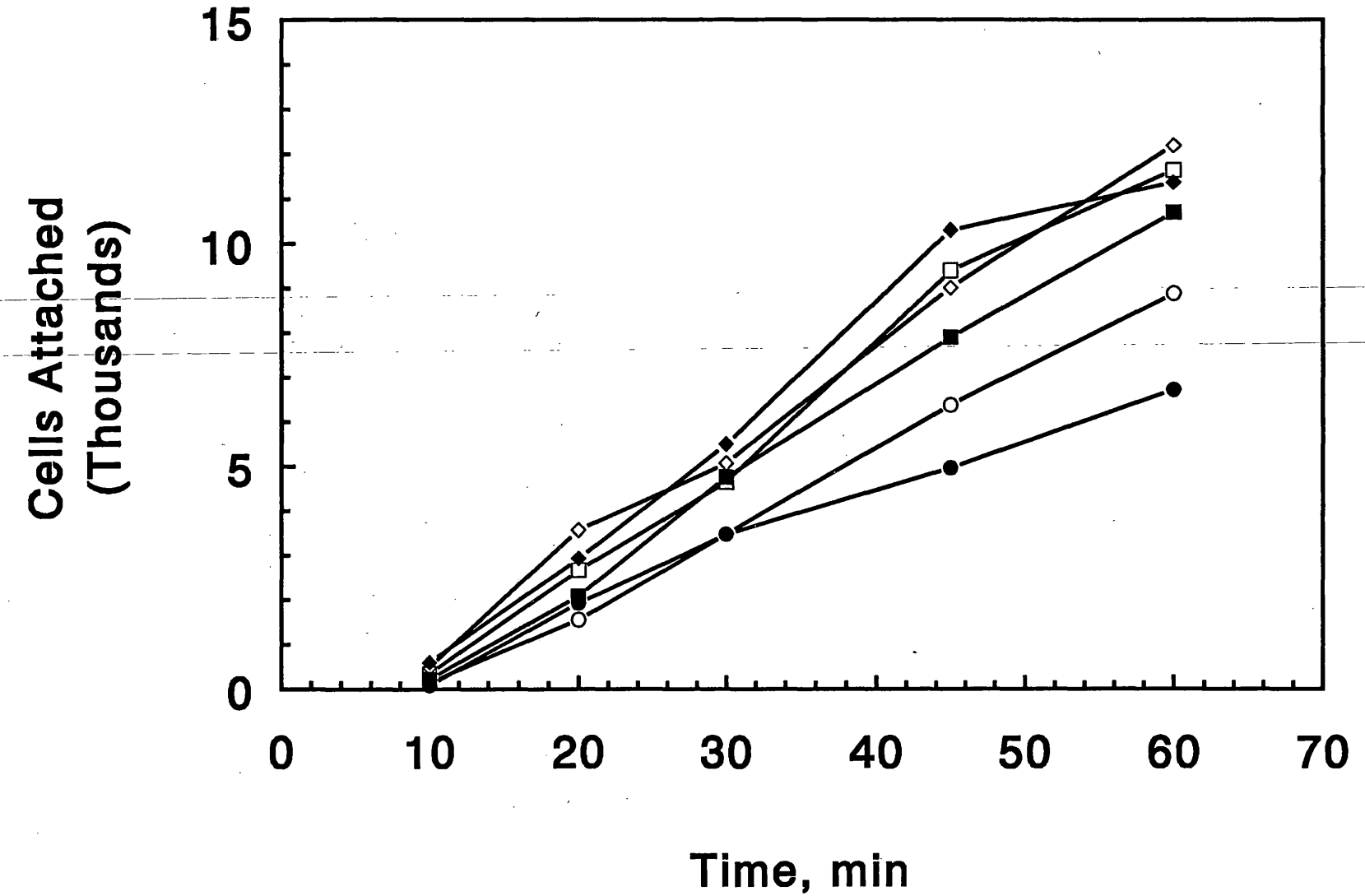

Cells Attached
(Thousands)
15
10
5
0
0
10
20
30
40
50
60
70
Time, min

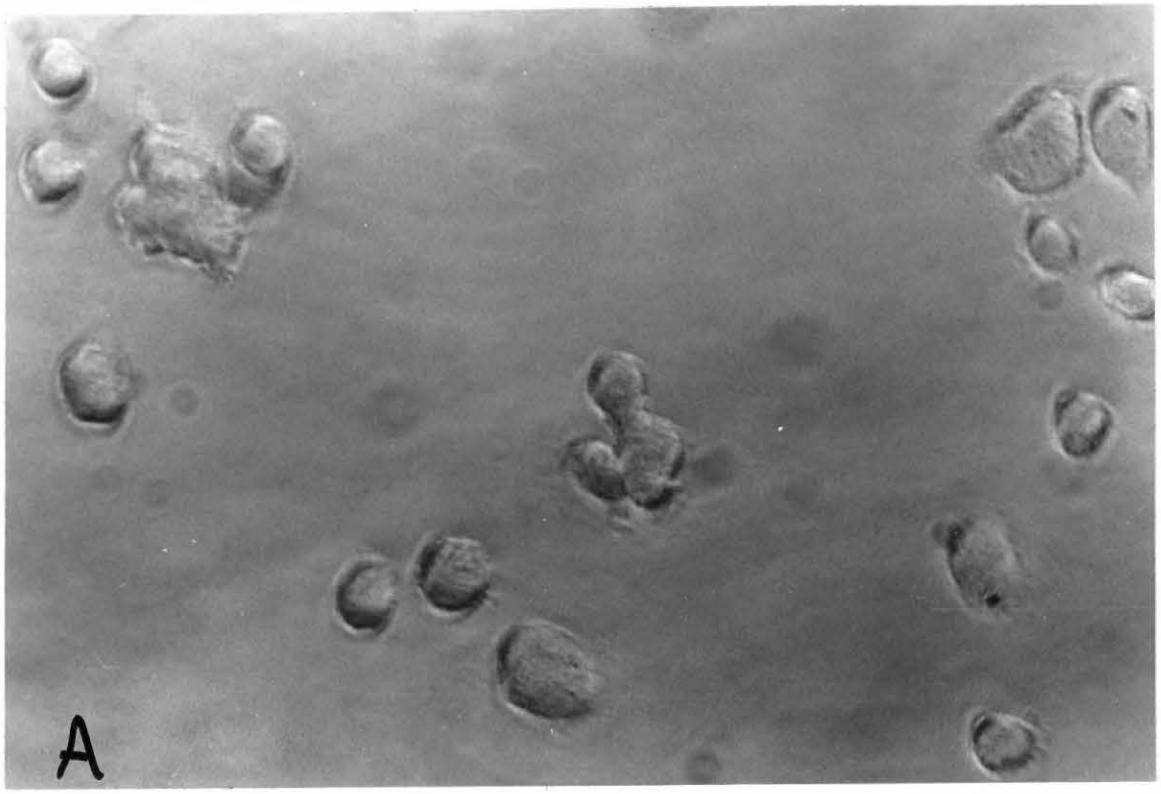
Table II. *Cells Attached[*] as a Function of Nicotine Concentration and Time of Exposure*

	Nicotine Concentration (μM)						
Time	0	0.025	0.05	0.1	0.2	0.4	p[**]
10 min	79 (141)	218 (141) NS[***]	346 (216) NS	598 (141) p<0.05	492 (298) p<0.05	492 (298) p<0.05	0.0232
20 min	1946 (487)	2105 (487) NS	2693 (389) NS	2958 (437) p<0.05	3488 (940) p<0.05	2408 (437) NS	0.0421
30 min	3511 (818)	4806 (487) NS	4702 (702) NS	5554 (818) p<0.05	5123 (878) NS	5710 (1266) p<0.05	0.1816 NS
45 min	5017 (389)	7990 (343) p<0.05	7990 (256) p<0.05	9501 (591) p<0.05	10,419 (487) p<0.05	9014 (759) p<0.05	<0.0001
60 min	6796 (538)	10,820 (646) p<0.05	11,775 (818) p<0.05	11,500 (702) p<0.05	12,332 (538) p<0.05	12,895 (538) p<0.05	<0.0001

[*]Cell Number by MTT Assay (± S.D.)
[**]ANOVA
[***]Fisher's LSD Test (NS = Not Significant)

Figure 4:

A. Control cells 30 minutes after plating. Cells appear raised with very little spreading (original magnification x 400).
B. Cells treated with 0.4 μM nicotine 30 minutes after plating. They appear more flattened than control cells (original magnification x 400).


A

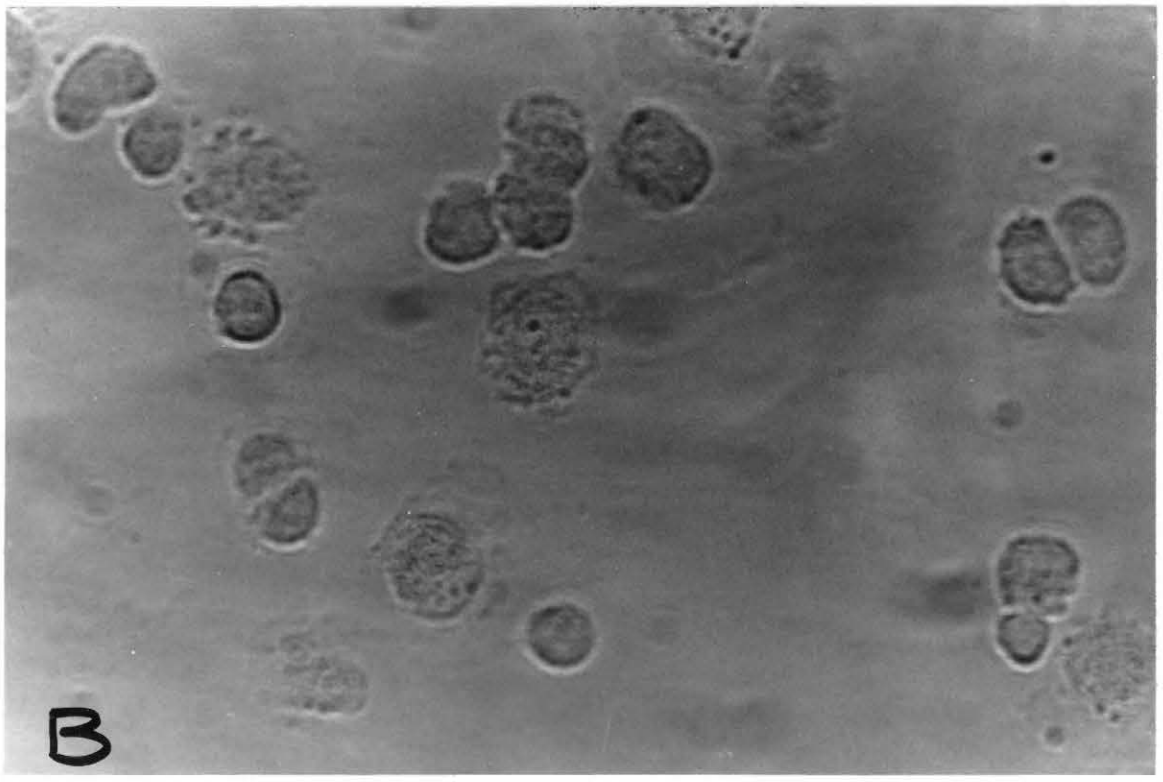
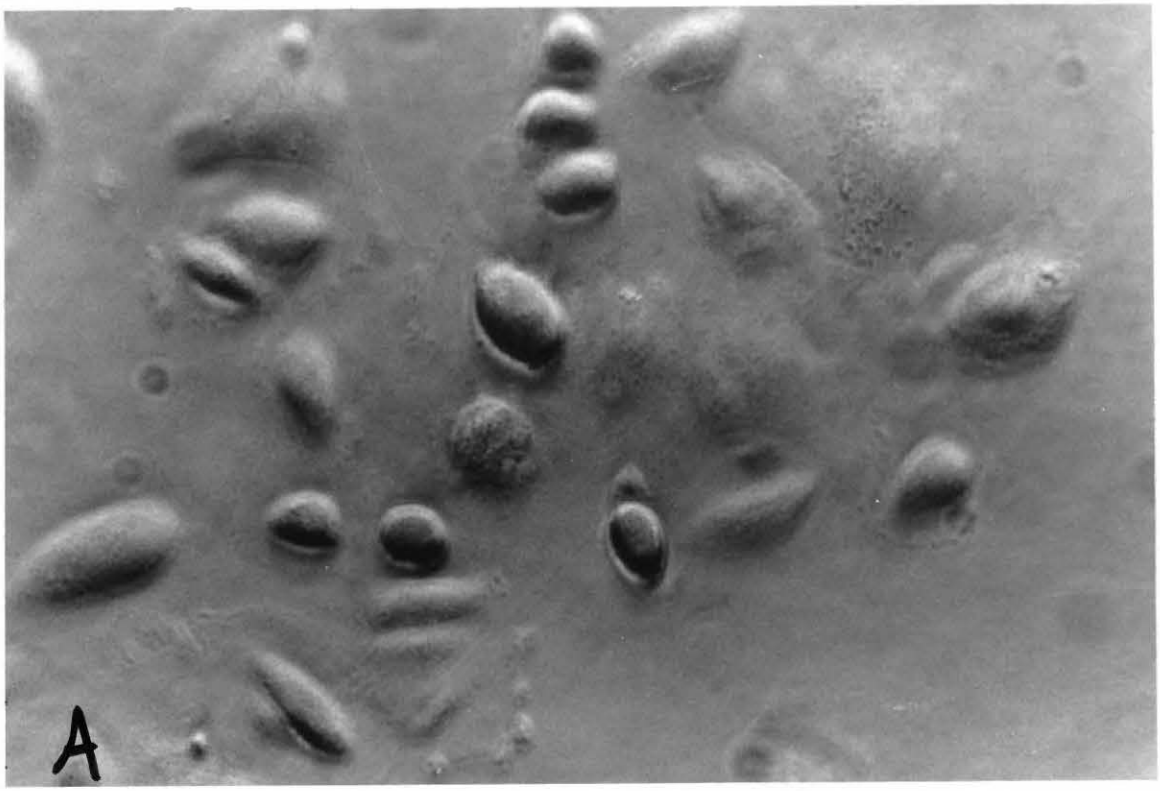
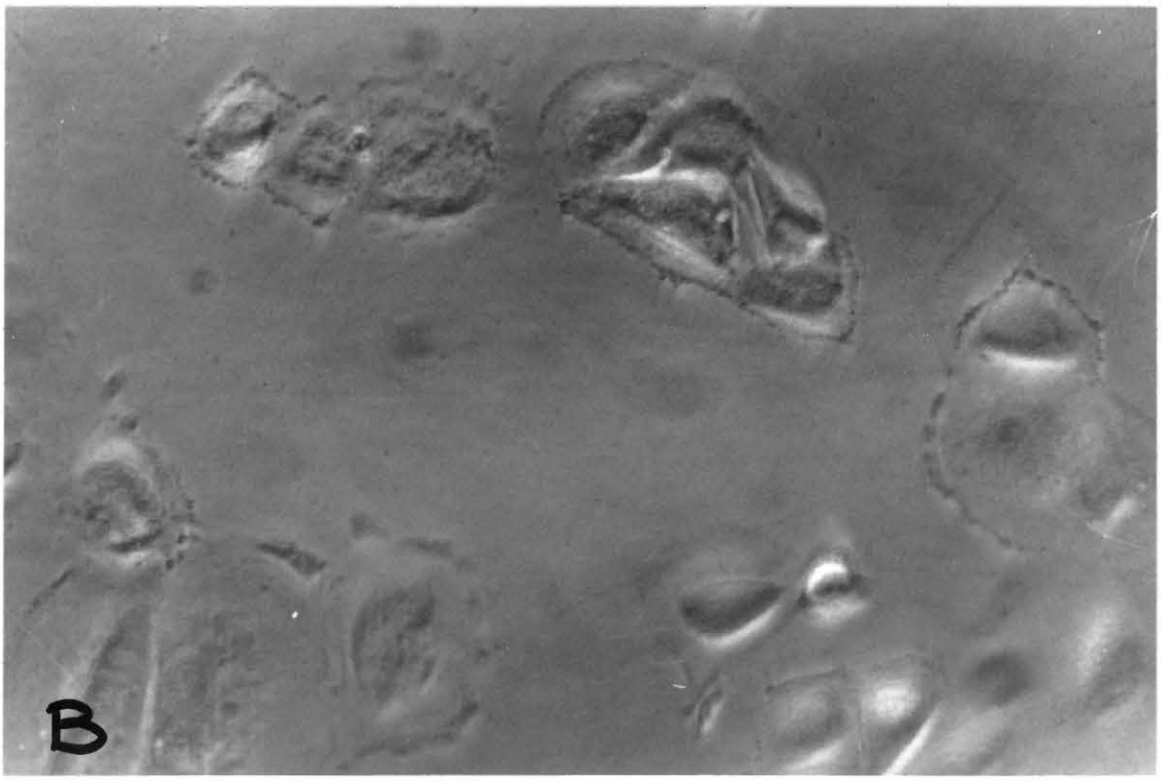

B

Figure 5:

A. Control cultures 60 minutes after plating. Cells appear more oval-shaped than at 30 minutes, but still raised from the surface with less spreading than nicotine-treated cells (original magnification x 400).

B. Cells treated with 0.4 μM nicotine 60 minutes after plating. There was almost complete flattening of the cells, and roughening of the margins was very evident (original magnification x 400).


A


B

After 48 hours of exposure to nicotine, there was a statistically significant increase in cell number for all concentrations (Figure 6). The lower level at 0.2 μM at 4 hours was not significantly different from control numbers and may reflect plating variability (Table 3).

Cell Growth Following a 1-Hour Pre-incubation with Nicotine:

Cells plated and allowed to attach overnight were refed with media containing various concentrations of nicotine. After 1 hour the media were removed and the cells refed with fresh media lacking nicotine in order to determine whether any responses persisted following nicotine removal. One hour after removal of nicotine and refeeding there was an initial increase in absorbance likely due to a burst of enzyme activity due to refeeding with fresh medium. Following the initial increase, the number of cells was significantly below those in control cultures (Figure 7). By 24 hours post-nicotine removal, cell numbers started to increase toward control values, but, by 48 hours, had not yet achieved that level. At all nicotine concentrations at 1, 20 and 24 hours after removal of nicotine, the cell numbers as determined by the MTT assay were significantly different from control values ($p < 0.05$) (Table 4). Cells that were pre-exposed to the 0.025 μM nicotine tended to rebound back toward control levels earlier than the other concentrations.

Reproduction Upon a 6-Hour Pre-incubation with Nicotine:

When cells were treated as above but with a 6-hour pre-incubation, the initial burst of activity was not detected. Rather, a decline, which continued until about 20 hours post-

Figure 6: *HGF reproduction with continuous nicotine exposure. Increases after 48 hours were significantly greater than control for all nicotine concentrations ($p < 0.05$).*

- ● *0.025 μM nicotine*
- ○ *0.05 μM nicotine*
- ■ *0.1 μM nicotine*
- □ *0.2 μM nicotine*
- ▲ *0.4 μM nicotine*
- \- - *Control*

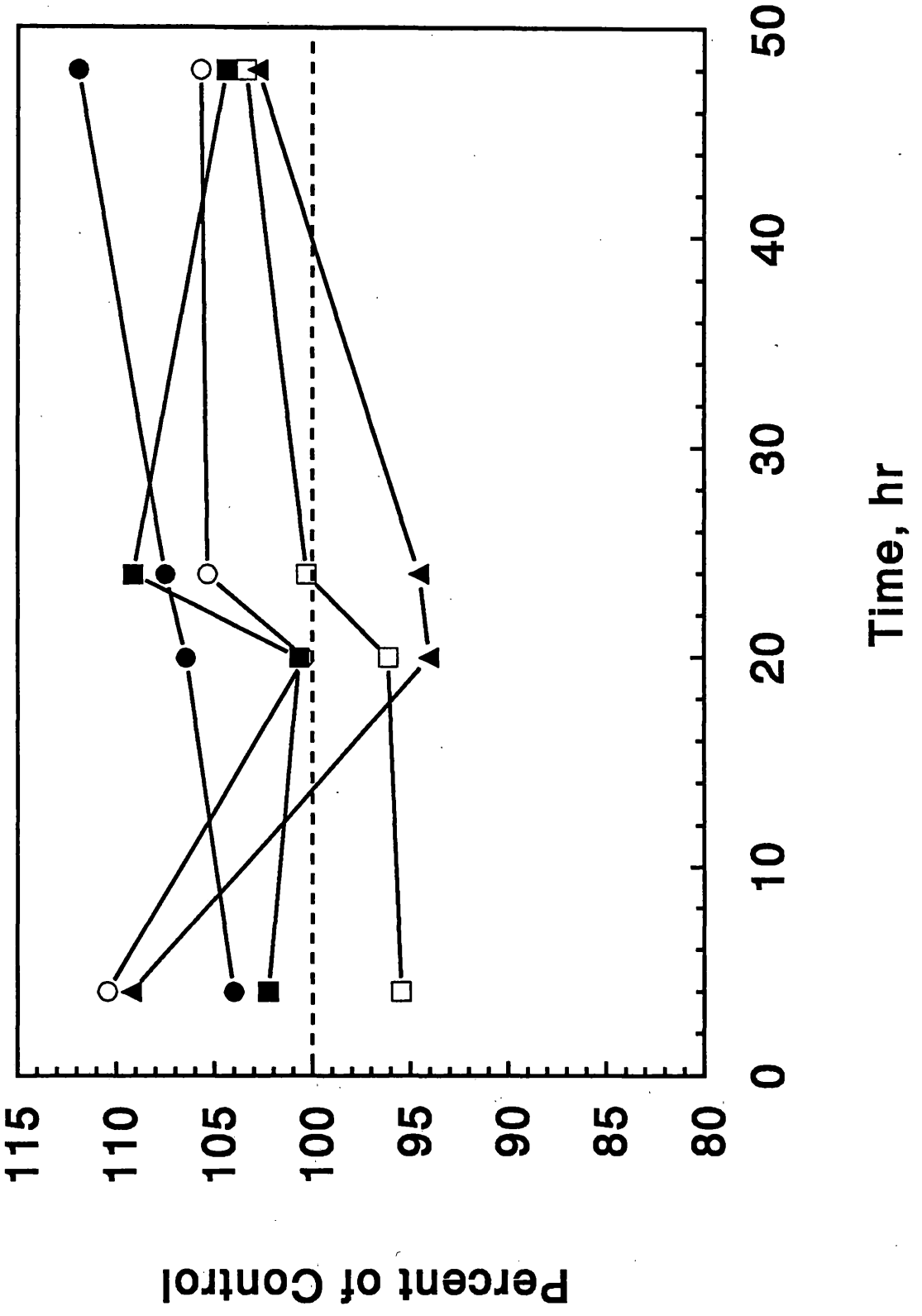

Percent of Control
115
110
105
100
95
90
85
80
0
10
20
30
40
50
Time, hr

Table III. *Cell Reproduction* Upon Continuous Nicotine Exposure*

	Nicotine Concentration (μM)						
Time	0	0.025	0.05	0.1	0.2	0.4	p**
4 hr	100% (3.9%)	104.1% (4.3%) NS***	110.4% (1.9%) p<0.05	102.3% (5.7%) NS	95.5% (3.7%) NS	109.3% (5.2%) p<0.05	<0.0001
20 hr	100% (4.3%)	106.4% (3.1%) p<0.05	100.4% (2.8%) NS	100.7% (2.7%) NS	96.1% (3.4%) p<0.05	94.0% (2.2%) p<0.05	<0.0001
24 hr	100% (3.7%)	107.0% (4.4%) p<0.05	105.3% (4.6%) NS	109.1% (5.3%) p<0.05	100.3% (4.7%) NS	94.5% (6.3%) NS	0.0001
48 hr	100% (3.2%)	112.6% (2.1%) p<0.05	106.8% (2.5%) p<0.05	106.4% (3.1%) p<0.05	106.1% (3.2%) p<0.05	106.5% (2.8%) p<0.05	<0.0001

*Percent of Control (0.00 μM) Activity (± S.D.)
**ANOVA
***Fisher's LSD Test (NS = Not Significant)

Figure 7: *HGF reproduction after a 1 hour pre-exposure to nicotine. At the 1-, 20- and 24-hour post-removal time periods, cell numbers for all nicotine concentrations were significantly different from control values ($p < 0.05$).*

- ● *0.025 μM nicotine*
- ○ *0.05 μM nicotine*
- ■ *0.1 μM nicotine*
- □ *0.2 μM nicotine*
- ▲ *0.4 μM nicotine*
- - - *Control*

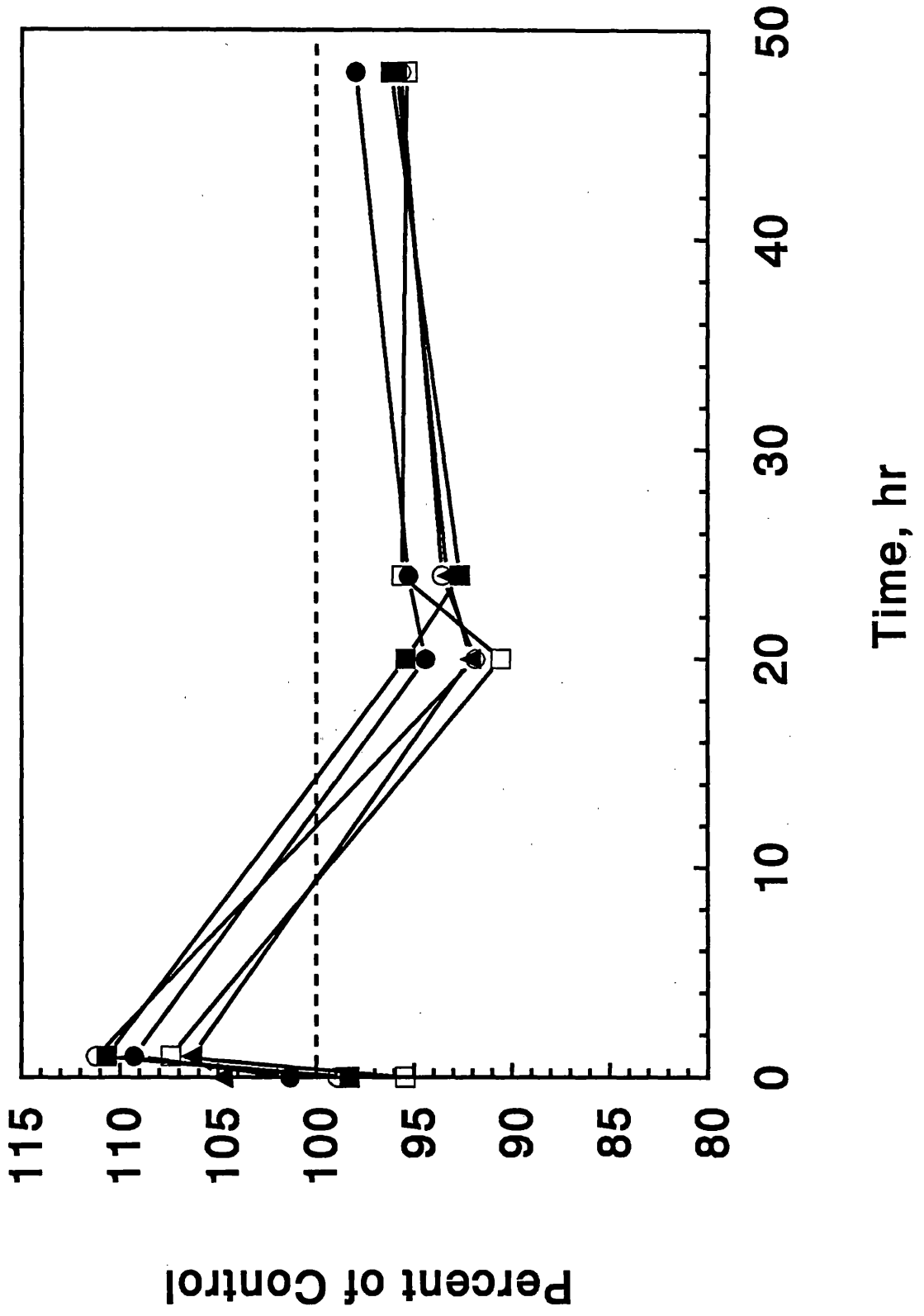

Percent of Control
115
110
105
100
95
90
85
80
0
10
20
30
40
50
Time, hr

Table IV. *Cell Reproduction* After a 1 Hour Pre-Exposure to Nicotine*

	Nicotine Concentration (μM)						
Time	0	0.025	0.05	0.1	0.2	0.4	p**
0 hr	100% (4.9%)	101.4% (3.5%) NS***	98.9% (3.9%) NS	98.3% (5.8%) NS	95.5% (5.8%) NS	104.8% (7.6%) NS	0.0351
1 hr	100% (3.6%)	109.3% (4.1%) p<0.05	111.3% (2.0%) p<0.05	110.7% (5.0%) p<0.05	107.4% (2.9%) p<0.05	106.4% (3.3%) p<0.05	<0.0001
20 hr	100% (2.4%)	94.4% (3.0%) p<0.05	91.9% (4.3%) p<0.05	95.4% (5.1%) p<0.05	90.6% (4.5%) p<0.05	92.1% (4.5%) p<0.05	0.0005
24 hr	100% (1.8%)	95.3% (3.8%) p<0.05	93.5% (2.9%) p<0.05	92.6% (3.0%) p<0.05	95.6% (2.9%) p<0.05	93.3% (1.5%) p<0.05	<0.0001
48 hr	100% (3.6%)	98.0% (4.6%) NS	95.7% (3.9%) p<0.05	96.2% (3.7%) NS	95.4% (3.5%) p<0.05	95.9% (2.7%) p<0.05	0.1371 NS

*Percent of Control (0.00 μM) Activity (± S.D.)
**ANOVA
***Fisher's LSD Test (NS = Not Significant)

removal, was noted. This was followed generally by some recovery (Figure 8). At 0 time at all nicotine concentrations the increase over control cultures was statistically significant ($p < 0.05$) (Table V). At 1 hour post-refeeding, cells exposed to all concentrations of nicotine showed activity above control values, but only those exposed to 0.4 μM were significantly elevated ($p < 0.05$). By 20 hours cells exposed to the lowest concentration of nicotine (0.025 μM) were still significantly elevated, while at other levels there was a decrease, with the higher concentrations (0.1, 0.2 and 0.4 μM) being significantly less than control values ($p < 0.05$). By 24 hours only the two highest concentrations were significantly different (below) from control values, and this pattern was still evident at 48 hours.

Summary of Results:

In summary, the results of this study indicate that (1) exposure to nicotine enhances human gingival fibroblast attachment to a substrate, and this appears to be concentration-dependent; (2) mitochondrial dehydrogenase activity is transiently decreased following nicotine exposure, but by 4 hours the enzyme activity is approximately equal to control; (3) exposure to low concentrations of nicotine has a significant stimulatory effect on HGF reproduction, while the higher concentrations produce a slight increase in HGF culture growth; and, (4) the effect of nicotine upon HGF reproduction does not seem to persist following nicotine removal.

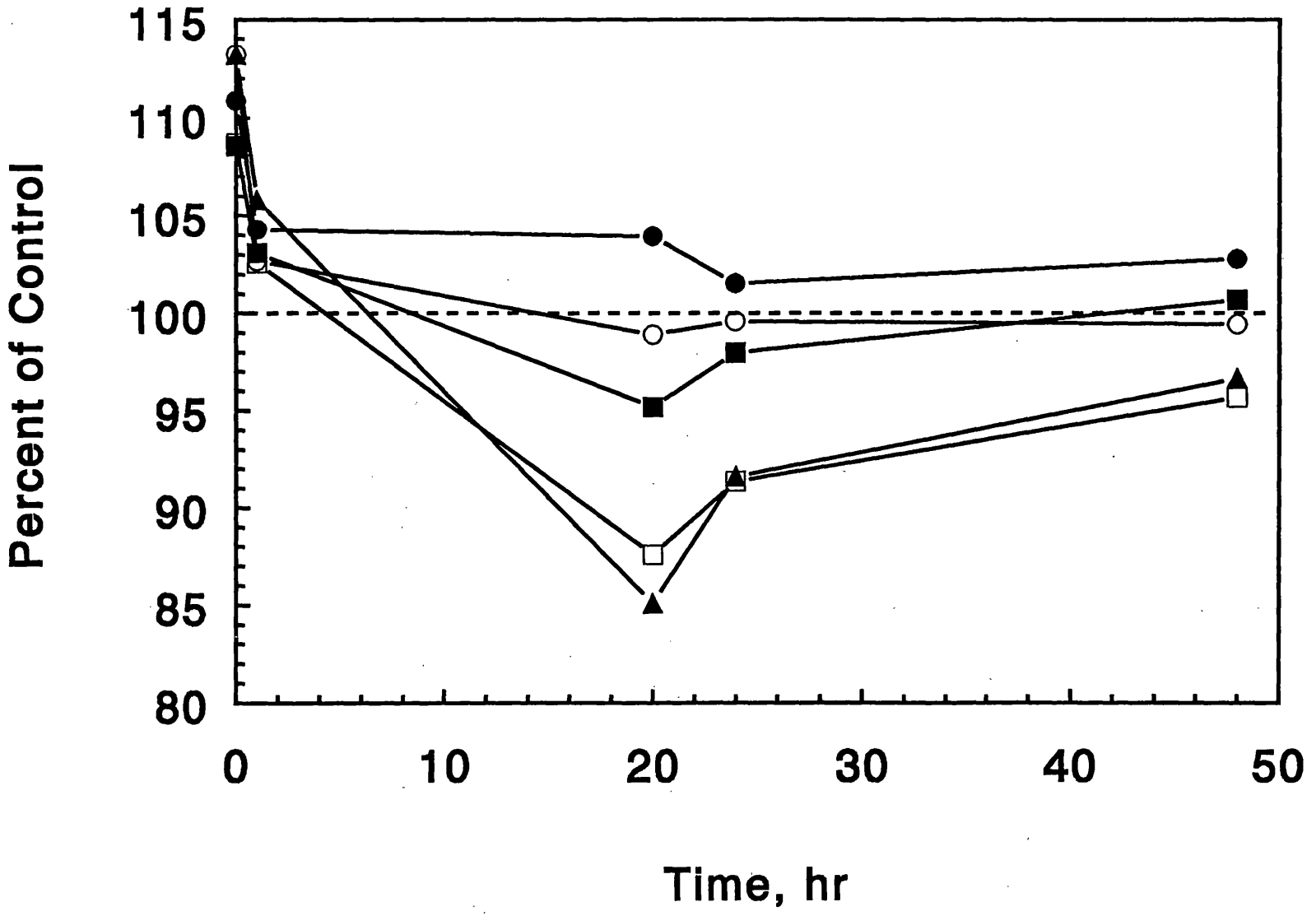

Percent of Control
115
110
105
100
95
90
85
80
0
10
20
30
40
50
Time, hr

Table V. *Cell Reproduction* After a 6 Hour Pre-Exposure to Nicotine*

	Nicotine Concentration (μM)						
Time	0	0.025	0.05	0.1	0.2	0.4	p**
0 hr	100% (4.9%)	110.8% (6.6%) p<0.05***	113.2% (9.0%) p<0.05	108.6% (6.7%) p<0.05	108.7% (7.0%) p<0.05	113.2% (4.8%) p<0.05	0.0107
1 hr	100% (4.6%)	104.3% (4.0%) NS	102.7% (4.1%) NS	103.1% (2.9%) NS	102.6% (5.3%) NS	105.8% (2.8%) p<0.05	0.3070 NS
20 hr	100% (2.7%)	104.0% (2.8%) p<0.05	98.9% (2.9%) NS	95.2% (4.3%) p<0.05	87.6% (4.5%) p<0.05	85.1% (3.6%) p<0.05	<0.0001
24 hr	100% (4.6%)	101.5% (6.0%) NS	99.6% (4.5%) NS	97.9% (6.3%) NS	91.3% (3.8%) p<0.05	91.6% (3.9%) p<0.05	0.0001
48 hr	100% (3.1%)	102.8% (2.9%) NS	99.4% (2.4%) NS	100.7% (2.4%) NS	95.7% (3.2%) p<0.05	96.6% (3.3%) p<0.05	0.0002

*Percent of Control (0.00 μM) Activity (± S.D.)
**ANOVA
***Fisher's LSD Test (NS = Not Significant)

DISCUSSION

Subsequent to the destruction caused by periodontal disease, the ability of fibroblasts to attach to root surfaces is a critical event. One of the major goals of periodontal therapy is the re-establishment of lost connective tissue attachment to the root surface. Recent studies have suggested a harmful effect of nicotine on fibroblasts, especially in regard to the reattachment and wound healing responses following periodontal treatment. In this study we demonstrate a positive effect of nicotine on gingival fibroblast attachment to a substrate; and, in concentrations to which light smokers are generally exposed, an enhanced rate of reproduction of gingival fibroblasts. Also noted in this study was that fibroblast adherence and growth may be differentially affected by the presence of nicotine, dependent upon time and concentration of nicotine both inside and outside the cells. Mitochondrial enzymatic activity was transiently depressed by nicotine, while simultaneously, increasing numbers of cells were seen attaching to a substrate.

The technique used in our study for enumerating fibroblasts involved a colorimetric method, the formation of formazan crystals from tetrazolium solution (MTT), which has been used in other studies to examine cell survival and proliferation (88-91). In the past, the most common method of measuring cell growth was the use of radiolabelled compounds, such as the incorporation of ^{3}H-thymidine into cellular DNA.

Although the ^{3}H-thymidine uptake assay may still be referred to as the "gold standard" for cell proliferation and cytotoxicity assays for some cell types, radioactive methods are highly technique sensitive, costly, and labor intensive. Also, DNA labeling by this method is cell cycle-dependent. This can lead to a high degree of variability within and between experiments, depending on the stage of the cycle when labeling occurs. Because only a fraction of the cells are labeled and thus detected, numbers may be over- or underestimated. In addition, large numbers of cells are required to obtain a level of labeling which is adequate to produce high counts. Thus, a method for detecting small changes in total viable cell numbers at any point in time is desirable for a study such as the one presented. The MTT colorimetric method for determining cell number avoids some of these problems and minimizes others. The procedure can be used in multiwell plates with small numbers of cells and read on an automatic scanning spectrophotometer with a high degree of precision. Mosmann (83) originally described the chemistry behind the tetrazolium salt technique, with other investigators refining the technique with several improvements (88-91). Tada et al (88) compared the results of the MTT assay versus ^{3}H-thymidine uptake in the growth of interleukin 2 (IL-2)-exposed cell samples and concluded that the difference between the two techniques in measuring IL-2-dependent T-cell lines was less than 5%. Denizot and Lang (89) tested the MTT procedure against ^{3}H-thymidine uptake in IL-2-dependent T-lymphoma cells and T-lymphocytes and found equal sensitivity among both procedures.

As previously mentioned, the MTT assay is an appraisal of viable cell number measured indirectly through the mitochondrial dehydrogenase activity of the cells. In

performing studies which assess the effect of a substance on cells, where the assay used measures a specific metabolic step, investigators must first determine the effect the experimental substance may have on that particular metabolic step. This was done for the assay used in these studies. In this case we measured cell growth and attachment by mitochondrial enzyme activity. As described below, the current studies showed that nicotine had a transient effect on the mitochondrial enzyme activity, but the assay was a good reflection of cell numbers at times after about 1 hour post-nicotine exposure. With a recognition of this transient alteration in enzyme activity, it also could be used to determine the effects of nicotine exposure on cell attachment at times less than 60 minutes post-exposure, as will be discussed later. Thus, the MTT method can replace the use of radioisotopes in many types of cell growth studies.

The study of human gingival fibroblast reproduction in the presence of tobacco components has not previously been done, although non-specific binding and uptake of nicotine by HGF have been demonstrated (39). The effects of nicotine on gingival fibroblast attachment also have not been studied, although Raulin et al (40) examined the effect of nicotine on foreskin fibroblast attachment utilizing microscopic methods. The present study shows that exposure to nicotine enhances gingival fibroblast attachment to a surface, and that this enhanced effect is concentration dependent. Raulin et al (40) concluded that foreskin fibroblasts exposed to nicotine exhibited structural alterations which might possibly prevent the cells from becoming "firmly attached" to root surfaces. Their assumptions were based on seeing an altered cell surface morphology under the scanning electron microscope. They suggested that these changes were associated with

a "disturbance in fibroblast attachment". Foreskin fibroblasts are from neonatal tissue and may behave differently from cells of periodontal origin from adults. Also, our attachment study was quantitative, in addition to using morphologic criteria. It showed a significant increase in HGF attachment with increasing nicotine concentration ($p < 0.0001$ for 45 and 60 minutes). The colorimetric assay used in the present study counts living cells by measuring those that are actively producing mitochondrial dehydrogenases, primarily succinic dehydrogenase. The enzyme activity study was performed to determine if the increase in attachment seen was a true increase in numbers of cells attaching, or if the nicotine was simply stimulating the production of mitochondrial enzymes. Instead of any enzyme stimulation, nicotine steadily inhibited mitochondrial dehydrogenase activity to as little as 22% of control at 45 minutes, with near recovery toward control values by 60 minutes. This type of response in the brief time period can be accounted for by changes in enzyme activity, as opposed to changes in cell numbers. This unexpected finding further strengthens the results, since the numbers of attached cells were increasing at the same time that the assay was measuring decreased enzyme activity. Thus the reported numbers of attached cells at these times are actually underestimated.

The morphological appearances seen during fibroblast adhesion and spreading on glass surfaces have been described in detail by Rajaraman et al (92). This process consists of cell attachment or adhesion, growth of filopodia in a centrifugal manner, web-like growth of the cytoplasm, and compression of the central part of the cell, with the fibroblast becoming more flattened. A distinguishing feature of the elongating filopodia are spherical tips of approximately 800-1600 Å diameter (92). These spherical tips are

thought to aid in cell attachment by decreasing the contact distance between cell and substratum. Examining the role that electrostatic forces may have in cell attachment to a substrate, Rajaraman et al (92) found that an increased surface negativity impedes the rate of cell spreading on a glass surface. Cell membranes of fibroblasts and tissue culture surfaces both have a net negative charge which may tend to make them repel each other. Nicotine, being positively charged, may act to overcome these repulsive forces which retard cell adhesion and spreading. The net effect could be attraction, or at least less repulsion; thus, nicotine would function as an attachment enhancer. This is not a novel idea, as many substances are used today in cell studies which enhance attachment through their being electropositive.

Another possible explanation for our attachment results is the role which the synthesis and secretion of cell adhesion proteins may play in mediating fibroblast adhesion and spreading. Although many of these cell adhesion molecules are glycoproteins such as the integrins and fibronectin, other families of attachment receptors include immunoglobulins, cadherins, lectin-EGF-complement cell adhesion molecules (LEC-CAM), and receptors that target lymphocytes (93). Integrin expression has been discovered in every cell type known; they help connect cells to their extracellular matrix and/or substratum (94). An increase in the rate of formation of any of these attachment proteins, including the collagens, might help in the anchorage and spreading of gingival fibroblasts. Still other adhesion molecules that may be important in the attachment of cells to a substrate are the proteoglycans, which are proteins with glycosaminoglycan side chains (95). In quantitative studies on fibroblast attachment, we are often seeing not only

an effect on the number of cells attaching compared to a control, but also indirectly we are examining how strong this attachment might be to a substrate. In the present study on attachment, the tissue culture well emptying and washing procedures may well have reduced the number of fibroblasts in the control group versus the nicotine-treated groups because the control cells were not as flat and in close contact to the substratum, or as adherent as the experimental cells. A fibroblast that is more settled on a surface would have a greater area of its exterior in contact with a substratum, and hence, less likely to be lost during manipulations. Our findings with the phase contrast microscope demonstrated a greater flattening of the nicotine-treated cells than the controls after one hour, lending credence to the theory of a stronger adhesive force in the experimentals. However, after four hours there appeared to be no difference in the morphology of the experimental and control cells. Thus, our results could actually be explained by a variety of different physicochemical events, any and/or all of which may play an important part in cell-to-substrate adhesion.

This study also demonstrated a stimulatory effect of nicotine on fibroblast proliferation, especially at low nicotine concentrations (0.025 μM) as seen in light tobacco users. Over a 48-hour period the cells continuously exposed to 0.025 μM nicotine steadily increased in number, exhibiting a near linear growth pattern. As the nicotine concentration increased, however, the cells decreased in number until 20 hours, and then started to increase up to 48 hours. After 48 hours of exposure to nicotine, the higher concentrations all showed a statistically significant increase in cell number compared to the control cells. These results seem to suggest that, except for the very low nicotine

concentrations perhaps equivalent to amounts seen in "light" smokers, the metabolism of cells that are continuously exposed to high levels of nicotine may become transiently impaired by these levels (or some cells may be killed by it) until the cells can sufficiently overcome this effect. As the cells recover from this effect, cellular metabolic activity is once again stimulated, resulting in an increased proliferation rate. Another possible explanation to consider is that replication in the presence of nicotine may be slowed due to alterations in the cell cycle. Also, as cells divide they may become more rounded, and in general become less adherent. This effect may be enhanced in the presence of nicotine and possibly lead to cell loss during cell manipulation.

The fibrobasts that were pre-incubated with nicotine for one hour showed a burst of metabolic activity after refeeding, but this stimulation did not seem to persist upon removal of the nicotine, although the cells were approaching control values 48 hours later. The decreased numbers at 20 and 24 hours may be an expression of cell loss due to residual toxicity by internalized nicotine. This possibility is further enhanced by the decreases seen at these same time points in cells continuously exposed to nicotine. The recovery by this latter group (continuously exposed cells) then could result from the cells avoiding the toxicity of internalized nicotine, perhaps by sequestering internalized nicotine within vesicles. Raulin et al (40) observed "vacuolization" in human foreskin fibroblasts upon long-term exposure to nicotine, and the degree of vacuolization seen increased as the nicotine concentration increased. These may actually have been vesicles containing nicotine.

The cells that underwent a six-hour pre-exposure period did not show the same initial burst of activity or number as did those that were pre-exposed to nicotine for only one hour. These cells also initially decreased in number, but after 20 hours post-removal of nicotine their numbers gradually increased toward control values. It is possible that initially while the cells were exposed to nicotine there was some stimulation of division until internal concentrations accumulated to an inhibitory level, analagous to the stimulation seen with continuous exposure to 0.025 μM nicotine, but a negative effect occurs as internal concentrations increase. Gradually the cells' metabolic activity may return as the nicotine is sequestered into vesicles and they again begin to multiply.

These findings concerning gingival fibroblast reproduction are not surprising considering the changes in oral tissues one usually sees in chronic smokers. Tobacco users frequently present with gingiva which has a very fibrotic appearance, possibly indicating cellular hyperplasia. These clinical findings that frequently are seen in smokers correlate well with our results on HGF proliferation. Additionally, our observations suggesting a stimulatory effect on HGF growth when exposed to low concentrations of nicotine also relate favorably to the lack of a negative effect on wound healing post-surgically reported by some clinicians in "light" cigarette smokers (15). On the other hand, as the cells exhibit a more rapid growth rate upon exposure to nicotine, it is possible that the aging process of the cells is also accelerated. This would help explain the diminished response to treatment that is often seen in tobacco users.

Although most published studies indicate that tobacco users are more prone to developing periodontal disease and/or having more severe disease, some authors attribute

the level of disease more to "changes in the oral cleanliness" than to smoking habits (4). Past investigations into the oral hygiene levels of smokers versus non-smokers are inconsistent at best, and the amount of bacterial plaque present in smokers compared to non-smokers seems to fluctuate from one clinical report to another. Several studies from our laboratories have recently attempted to focus on the role of nicotine and its major metabolite, cotinine, as an etiological factor in periodontal disease (40,96,97). McGuire et al (96) found the presence of cotinine in saliva and gingival crevicular fluid of chronic cigarette smokers, with no cotinine detected in non-smokers. Cotinine is also present in the smoke of burnt tobacco, although this substance has no significant pharmacological activity (69). Cuff et al (97) discovered the presence of nicotine in much higher concentrations on root surfaces of periodontally diseased teeth, and that root planing removed the nicotine from the root surfaces. Whereas most of the literature considers smoking and tobacco use as a secondary factor in the disease process, there is no experimental evidence available that can support the claim that nicotine definitely plays a part in the etiology of periodontal disease. Our results lend credence to this opinion. Other agents that may be present in tobacco need to be examined also.

Most published studies looking at the effects of tobacco products on cells of the periodontium involve nicotine. Although nicotine constitutes a major portion of the tobacco leaf, other products in tobacco smoke are worthy of further study concerning their biological effects. Specific nitrosamines are found in tobacco including several proven carcinogens. Isolating the many different components of tobacco smoke would be a

formidable task, but other constituents of the different phases of smoke must be examined in order to explain the correlation seen between periodontal disease and tobacco usage.

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