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CYTOTOXIC AND CYTOSTATIC EFFECTS OF ARECOLINE AND SODIUM NITRITE ON HUMAN CELLS *IN VITRO*

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Arecoline, an active alkaloid of *Areca catechu L.*, and sodium nitrite, a food additive, are highly cytotoxic and cytostatic on the Hep 2 cell line when administered in an acidic environment (pH 4.2) in the presence of S-9 mixture. Hep 2 cells (10^6) were treated with either 0.145, 0.725 or 1.449 mM sodium nitrite or 0.042, 0.085 or 0.339 mM arecoline or sodium nitrite (0.145 mM) plus varied concentrations of arecoline (0.042, 0.085 or 0.339 mM). Their effects were additive in nature. Hep 2 cells exposed to this combination showed reduced cell survival, and lower rates of DNA and protein syntheses. Involvement of N-nitroso derivatives of arecoline is suggested to explain the results. On the basis of these studies, we speculate that N-nitroso compounds derived from arecoline can (weakly) interact with DNA.

Human beings are constantly exposed to a heterogeneous variety of chemicals in their daily life. Betel nut *(Areca catechu. L.)*, used as a masticatory product by a large population of India, many South-East Asian countries and Latin America, is a potent source of various alkaloids (IARC, 1985). Similarly, sodium nitrite ($NaNO_2$, a widely used food additive), finds its way into the human body through several food and drink preparations. $NaNO_2$ forms nitrous acid in an acidic environment and is thought to possess mutagenic effects in cultured mammalian cells (Kodama *et al.*, 1976). On the other hand, the major alkaloid of betel nut, arecoline, under mild nitrosation conditions, forms N-nitroso compounds (NOCs) *in vitro* (Wenke and Hoffman, 1983; Nair *et al.*, 1985) and *in vivo*, and is implicated in carcinogenesis (IARC, 1985; Castegnaro, 1988). Saliva samples of betel-quid/betel-nut chewers show appreciable concentrations of betel-nut-specific nitrosamines (BSNA) and tobacco-specific nitrosamines (TSNA) (Wenke and Hoffman, 1983; Wenke *et al.*, 1984; Nair *et al.*, 1985, 1986). Similarly, urine samples of betel-nut chewers also show presence of BSNA (Ohshima *et al.*, 1989). The betel quid and its ingredients have been shown to be mutagenic using the Ames test (Shirname *et al.*, 1983) and mammalian gene mutation tests (Shirname *et al.*, 1984). Cytotoxic and genotoxic effects of the components of betel nut have also been noted by Sundquist *et al.* (1989). Other investigations along these lines have revealed that, upon ingestion of nitrate together with extracts of betel nut in the presence of proline, endogenous nitrosation is inhibited (Stich *et al.*, 1983), indicating the possibility of nitrosation of betel-nut constituents. The presence of $NaNO_2$ in food and drink preparations may enhance the formation of NOCs from arecoline in the mouth and the adjoining parts of the digestive tract which may thus react with biomolecules to induce pathogenic conditions, including carcinogenic transformation. Furthermore, a report on gastric juice pH, nitrite, and gastric cancer in a South African population (Steenkamp *et al.*, 1989) indicates that an acidic pH favors accumulation of nitrite in the gut and consequently facilitates carcinogenic transformation. It has been postulated that arecoline may undergo NOC formation at an enhanced rate in the presence of a nitrite (IARC, 1985; Ohshima, 1989). Therefore, it would be relevant to investigate the effects of these 2 compounds in mammalian cells. We have previously observed that the aqueous extract of betel nut apparently contains some additional factors to initiate transformation *in vitro* (Wary and Sharan, 1988). We now report a more detailed investigation of the betel nut alkaloid, arecoline, in the presence of $NaNO_2$.

MATERIAL AND METHODS

Chemicals

All chemicals were of analytical grade and were used without further purification. All solutions were prepared in deionized double-distilled water.

Cell line

The Hep 2 cells were derived from a human carcinoma of the larynx, which was a gift from Dr. A.K. Prasad, Delhi University, at passage 76. The cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 15% fetal calf serum, penicillin-G (100 units/ml), and streptomycin (100 μg/ml). The cells were subcultured at 1:4 splits every 4th day. The cells were maintained at 37°C in 5% CO_2 and 80% humidity.

Preparation of tested chemicals

Appropriate concentrations of stock solutions of arecoline and $NaNO_2$ were prepared in phosphate-buffered saline. The Hep 2 cells were exposed to 0.145, 0.725 and 1.449 mM $NaNO_2$ or to 0.042, 0.085 and 0.339 mM of arecoline per culture vessel. The stock solutions of test chemical(s) were mixed with the culture medium (with S-9 mixture at pH 4.2) to obtain the desired chemical concentrations of exposure to the Hep 2 cells. To monitor the combined effect of these 2 tested chemicals, we prepared 3 combinations: a fixed 0.145 mM of $NaNO_2$ with either 0.042, 0.085 or 0.339 mM of arecoline per culture vessel.

Preparation of S-9 mixture

S-9 mixture [S-9] was prepared fresh from livers of Swiss albino mice according to McCann *et al.* (1975). The protein concentration was 24 mg/ml as determined by Bradford's method (1976).

Treatment of Hep 2 cells to test chemicals

Exponentially growing cells (10^6) under normal culture conditions were exposed to the test chemicals at a pH of 4.2. For this purpose, the appropriate test chemical concentrations were prepared in normal medium containing S-9 (0.2 ml) and the pH was adjusted to 4.2. The medium in which the cells were growing was then replaced by this treatment medium (pH 4.2) containing the test chemical(s). After 30 min, the treatment medium was removed, the cells were washed with Hanks' balanced salt solution and returned to normal culture medium. Various assays were carried out from this time onwards. The control cells were grown in media either at (i) pH 7.2 or at (ii) pH 4.2 in the presence of S-9 but without test chemicals.

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Cell population counting

The cells were counted in a hemocytometer by the dye exclusion technique (Wary and Sharan, 1988).

Molecular synthesis assays

Macromolecular synthesis assays were carried out at various time intervals after re-establishment of the cells in normal medium, free of test chemical(s). The cells were pulsed for 10 min with ^{14}C-Thymidine (^{14}C-TdR, 1 μCi/ml) to monitor DNA synthesis or ^{14}C-Leucine (1 μCi/ml) for protein synthesis. Labelling of the cells was terminated by washing with Hanks' balanced salt solution. The cells were scraped into a buffer (0.15 M sodium chloride and 0.15 M sodium citrate) and filtered through GF/D glass microfibre filters moistened with cold 5% TCA. The filters were washed thrice with cold 5% TCA (30 ml) and dried. The residual radioactivity (cpm) on the filters was monitored in a Beckman Liquid Scintillation Counter (LS-7000) using a toluene based cocktail.

DNA synthesis inhibition test

Measurement of the rate of DNA synthesis [percentage of control of incorporated ^{3}H-TdR in the DNA of Hep 2 cell following termination of 30 min treatment with test chemical(s) at pH 4.2 and in the presence of S-9] was carried out according to Painter (1977) with minor modifications. In short, after exposure to test chemical(s) for 30 min, the cells were pulsed with ^{3}H-TdR (5 μCi/ml) in MEM for 10 min. The cells were allowed to grow in the normal culture medium for up to 3 hr. The amount of radiolabelled DNA was determined in a Beckman Liquid Scintillation Counter (LS-7000).

RESULTS

Cell survival

Figure 1 shows the normal cell viability (at pH 7.2 without S-9) and at pH 4.2 (with S-9) as well as the cell numbers at various time intervals following 30-min treatments with $NaNO_2$ (0.145, 0.725 or 1.449 mM), or arecoline (0.042, 0.085 or 0.339 mM), or combinations of $NaNO_2$ (0.145 mM) and arecoline (0.042, 0.085 or 0.339 mM). All cell counts were carried out at approximately the same time. Very high cytotoxicity was observed beyond 0.339-mM doses of arecoline (B), 0.725-mM doses of $NaNO_2$ (A), and for all doses of arecoline + $NaNO_2$ (C) leading to cell death. Decreasing pH from 7.2 to 4.2 in the absence of S-9 was also extremely inhibitory to Hep 2 cell growth (result not shown).

Rate of DNA synthesis

Figure 2 shows DNA synthetic activity as a function of time at pH 7.2, at pH 4.2 in the presence of S-9 and following exposure of cells to $NaNO_2$, or arecoline, or to their combinations at pH 4.2 (see Figure legend for details).

Rate of protein synthesis

Figure 3 shows protein synthetic activity for Hep 2 cells at pH 7.2, at pH 4.2 in the presence of S-9 and following exposure of cells to various concentrations of $NaNO_2$, or arecoline, or their combinations at pH 4.2 (see Figure legend for details).

DNA synthesis inhibition test

Inhibition of DNA synthesis in Hep 2 cells as a result of exposure to combinations of $NaNO_2$ and arecoline is depicted in Figure 4. There was a rapid recovery in the rate of DNA

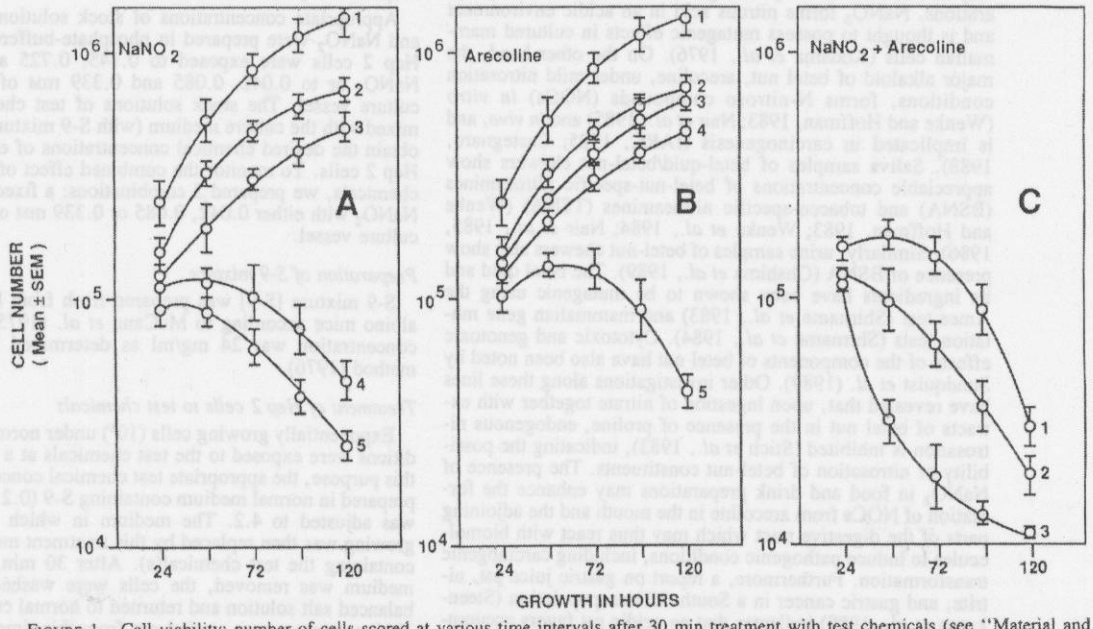

FIGURE 1 – Cell viability: number of cells scored at various time intervals after 30 min treatment with test chemicals (see "Material and Methods" for details). *(a)* Sodium nitrite. Curve 1: normal medium at pH 7.2; curve 2: normal medium with S-9 at pH 4.2; curves 3, 4, and 5: normal medium with S-9 at pH 4.2 with 0.145, 0.725 and 1.449 mM of $NaNO_2$, respectively. *(b)* Arecoline. Curve 1: normal medium at pH 7.2; curve 2: normal medium with S-9 at pH 4.2; curves 3, 4, and 5: normal medium with S-9 at pH 4.2 with 0.042, 0.085 and 0.339 mM of arecoline, respectively. *(c)* Combination of a fixed concentration of $NaNO_2$ (0.145 mM) plus 0.042, 0.085 and 0.339 mM of arecoline in curves 1, 2, and 3, respectively, in a normal medium with S-9 at pH 4.2. Each data point represents results from 6 independent experiments.

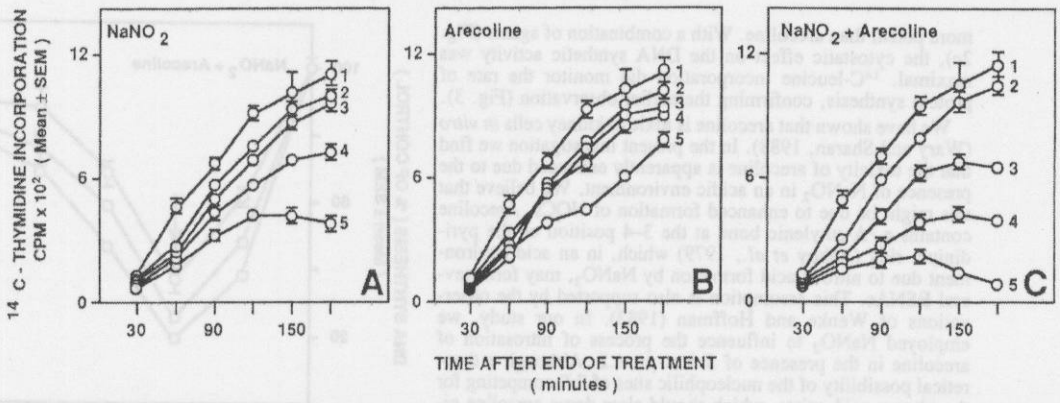

FIGURE 2 – DNA synthesis. The rate of DNA synthesis in the cells was monitored at various time intervals after 30 min treatment with test chemicals (see "Material and Methods" for details). Curve 1: normal medium at pH 7.2; curve 2: normal medium with S-9 at pH 4.2. *(a)* Sodium nitrite. Curves 3, 4, and 5: normal medium with S-9 at pH 4.2 with 0.145, 0.725 and 1.449 mM $NaNO_2$, respectively. *(b)* Arecoline. Curves 3, 4, and 5: normal medium with S-9 at pH 4.2 with 0.042, 0.085 and 0.339 mM arecoline, respectively. *(c)* Combination of a fixed concentration of $NaNO_2$ (0.145 mM) plus 0.042, 0.085 and 0.339 mM of arecoline. Curves 3, 4, and 5, respectively: normal medium with S-9 at pH 4.2. Each data point represents results of at least 4 independent experiments.

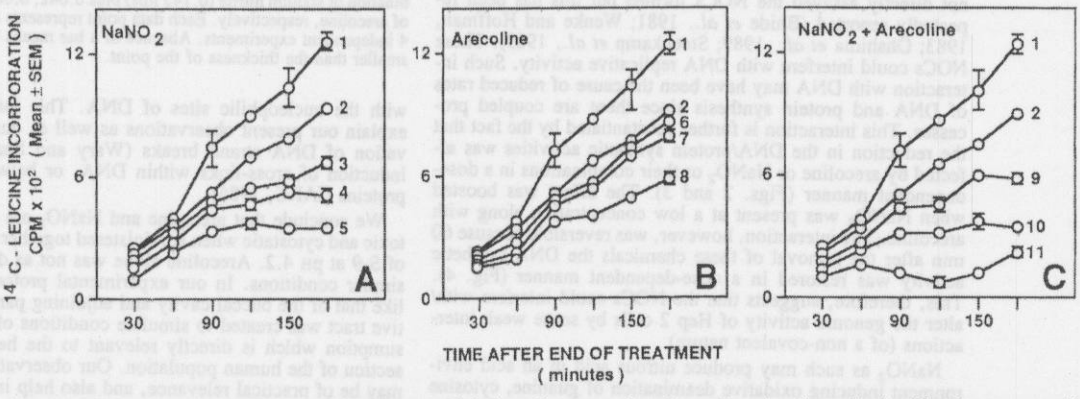

FIGURE 3 – Protein synthesis. The rate of protein synthesis in the cells was monitored at various time intervals after 30 min treatment with test chemicals (see "Material and Methods" for details). Curve 1: normal medium at pH 7.2; curve 2: normal medium with S-9 at pH 4.2. *(a)* Sodium nitrite. Curves 3, 4, and 5: normal medium with S-9 at pH 4.2 with 0.145, 0.725 and 1.449 mM of $NaNO_2$, respectively. *(b)* Arecoline. Curves 6, 7, and 8: normal medium with S-9 at pH 4.2 with 0.042, 0.085 and 0.339 mM arecoline, respectively. *(c)* Combination of a fixed concentration of $NaNO_2$ (0.145 mM) plus 0.042, 0.085 and 0.339 mM of arecoline. Curves 9, 10, and 11, respectively: normal medium with S-9 at pH 4.2. Each data point represents results of at least 4 independent experiments.

synthesis from 60 min (arrow) onwards after removal of the test chemical(s). At the end of 3 hr, recovery in the rate of DNA synthesis was over 90%.

DISCUSSION

In an effort to understand betel-nut-induced carcinogenesis (Wary and Sharan, 1988, 1989; Wary, 1989) we have studied here one of the major alkaloids of betel nut, arecoline, choosing an acidic environment (pH 4.2) to mimic the acidic conditions of the buccal cavity (Stich *et al.*, 1986) and the adjoining parts of the digestive tract. Nitrite concentration and pH are known to affect the process of chemical carcinogenesis (Cohen, 1987; Steenkamp *et al.*, 1989). In this report we took arecoline with $NaNO_2$, a common food additive, and used an acidic pH to better understand the arecoline-induced damage. $NaNO_2$ was chosen because it forms nitrous acid in an acidic environment (as in the buccal cavity and the adjoining parts of the digestive tract) and enhances the process of nitrosation which supposedly forms potent carcinogens (IARC, 1985; Kodama *et al.*, 1976; Castegnaro, 1988). S-9 was added to bring the *in vitro* system close to the *in vivo* situation. Hep 2 cell growth was markedly inhibited when pH was reduced from 7.2 to 4.2 in the absence of S-9. In the presence of S-9, however, it was not. Hence, for all the experiments at pH 4.2, the S-9 mixture always remained present.

The Hep 2 cells had a reduced metabolism when the pH of normal culture medium was reduced from 7.2 to 4.2 as evidenced by reduced survival (Fig. 1) and reduced rates of DNA (Fig. 2) and protein (Fig. 3) synthesis. In all cases, reduction was significant. From the nature of the curves (Fig. 1), it is apparent that $NaNO_2$ enhances arecoline cytotoxicity since even the lowest concentration of arecoline (0.042 mM) caused noticeable cell death (Fig. 1*c*). Our results on ^{14}C-TdR incorporation show that both arecoline (Fig. 2*b*) and $NaNO_2$ (Fig. 2*a*) have cytostatic effects on DNA synthesis, nitrite being

more potent than arecoline. With a combination of agents (Fig. 2*c*), the cytostatic effect on the DNA synthetic activity was maximal. ^{14}C-leucine incorporation did monitor the rate of protein synthesis, confirming the earlier observation (Fig. 3).

We have shown that arecoline is toxic to kidney cells *in vitro* (Wary and Sharan, 1988). In the present investigation we find that the toxicity of arecoline is apparently enhanced due to the presence of $NaNO_2$ in an acidic environment. We believe that this might be due to enhanced formation of NOCs. Arecoline contains a $^3\Delta$-ethylenic bond at the 3–4 position on the pyridinium ring (Ashby *et al.*, 1979) which, in an acid environment due to nitrous acid formation by $NaNO_2$, may form several BSNAs. This assumption is also supported by the observations of Wenke and Hoffman (1983). In our study, we employed $NaNO_2$ to influence the process of nitrosation of arecoline in the presence of S-9 at pH 4.2. Although a theoretical possibility of the nucleophilic sites of S-9 competing for the nitrous acid exists, which should slow down arecoline nitrosation, our results show that significant amounts of NOCs could still be formed. Similarly, serum present in the medium may also interact with arecoline or $NaNO_2$. However, the possibility is low due to the acidic pH of the medium. Nonetheless, in our experimental protocol, formation of significant amounts of NOCs is apparent from our results (Figs. 2 and 3). We have not directly assayed the NOCs formed but this has been repeatedly reported (Bhide *et al.*, 1981; Wenke and Hoffman, 1983; Ohshima *et al.*, 1989; Steenkamp *et al.*, 1989). These NOCs could interfere with DNA replicative activity. Such interaction with DNA may have been the cause of reduced rates of DNA and protein synthesis since these are coupled processes. This interaction is further substantiated by the fact that the reduction in the DNA/protein synthetic activities was affected by arecoline or $NaNO_2$ or their combinations in a dose-dependent manner (Figs. 2 and 3). The effect was boosted when $NaNO_2$ was present at a low concentration along with arecoline. The interaction, however, was reversible because 60 min after the removal of these chemicals the DNA synthetic activity was restored in a dose-dependent manner (Fig. 4). This, therefore, suggests that the NOCs could interfere with/alter the genome activity of Hep 2 cells by some weak interactions (of a non-covalent nature).

$NaNO_2$ as such may produce nitrous acid in an acid environment inducing oxidative deamination of guanine, cytosine and adenine; protonated forms of nucleosides or nucleotides have been found to be more labile to deamination attack due to nitrous acid at pH 3.75 (Singer and Grunberger, 1983). The optimal pH for $NO_2^- \rightarrow HNO_2$ is known to occur in human stomach (Singer and Grunberger, 1983). In such a situation, $NaNO_2$ and arecoline probably react with each other to produce several NOCs of arecoline which are capable of interacting with the nucleophilic sites of DNA. This interaction could explain our present observations as well as our earlier observation of DNA strand breaks (Wary and Sharan, 1988) or induction of cross-links within DNA, or between DNA and proteins (IARC, 1985).

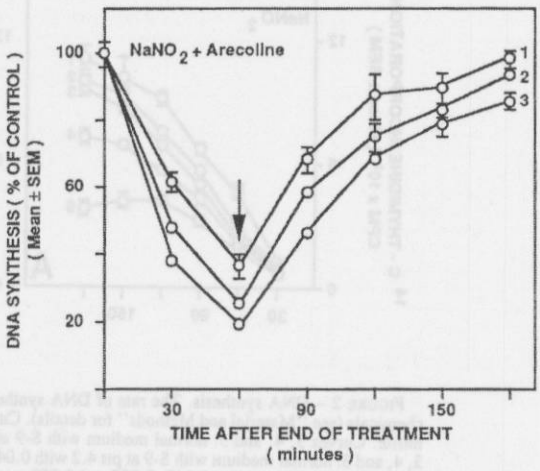

FIGURE 4 – DNA synthesis inhibition test. Curves 1, 2 and 3 show rates of DNA synthesis expressed as percentage of control in Hep 2 cells at various time intervals after the end of treatment with a combination of sodium nitrite (0.145 mM) plus 0.042, 0.085 and 0.339 mM of arecoline, respectively. Each data point represents results of at least 4 independent experiments. Absence of a bar means that the SEM was smaller than the thickness of the point.

We conclude that arecoline and $NaNO_2$ are intensely cytotoxic and cytostatic when administered together in the presence of S-9 at pH 4.2. Arecoline alone was not as damaging under similar conditions. In our experimental protocol, a situation like that of the buccal cavity and adjoining parts of the digestive tract was created to simulate conditions of betel nut consumption which is directly relevant to the health of a large section of the human population. Our observations, therefore, may be of practical relevance, and also help in understanding the damage caused by betel-nut alkaloid, arecoline.

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