

Aqueous extract of betel-nut of North-East India induces DNA-strand breaks and enhances rate of cell proliferation in vitro*

Effects of betel-nut extract in vitro

K. K. Wary and R. N. Sharan

Radiation and Molecular Biology Unit, Department of Biochemistry, School of Life Sciences, North-Eastern Hill University, Shillong 793014 India

Summary. An aqueous extract of betel-nut has been found to be able to induce strand breaks in DNA of mouse kidney cells in vitro. It has been also found to be able to enhance the rate of cell division at a dose of 100 µg/ml while a higher dose of 250 µg/ml was extremely toxic to the cells. Compared with arecoline (10 µg/ml), the aqueous extract of betel-nut seems to be a more potent carcinogen to mouse kidney cells in vitro.

Key words: Betel-nut – DNA-strand breaks – Carcinogenesis – In vitro

Introduction

In the North-Eastern part of India, betel-nut (*Areca catechu* L.) is normally chewed with betel leaf (*Piper betle* L.) and a little slaked lime. This results in characteristically red saliva and teeth. The habit of betel-nut chewing and occasional swallowing is associated with oropharyngeal cancers in humans (Muir and Kirk 1960; Ashby et al. 1979; IARC 1985) and accounts for over 50% of the total cancer incidence in India (Senghvi 1981). The Khasi tribe of North-Eastern India chew raw and unprocessed betel-nut (locally known as "Kwai") as part of the normal social custom. This betel-nut consumption shows an immediate thermogenic physiological response, lasting 2–3 min, with significant perspiration on the forehead. This is markedly different from the effect of processed betel-nut consumed elsewhere in the world, especially in Southeast Asian countries (see IARC 1985). Various

constituents of betel-nut have been shown to induce mutation, sister chromatid exchange and transformation (Umezawa et al. 1981; for review see IARC 1985). From this region a higher frequency of occurrence of micronucleated cells in buccal mucosa of people who chew betel-nuts has been reported (Stich et al. 1982; Stich et al. 1983). Chromosome-damaging activity has also been reported in the saliva of people who chew betel-nuts (Stich and Stich 1982). It has also been found that betel-nut can induce an increased rate of mutation in *Salmonella typhimurium* (Shirname et al. 1983).

In the process of carcinogenesis, DNA is the prime target for various chemicals and agents. Betel-nut, which is rich in alkaloids (Arjungi 1976; Ashby et al. 1979; Sharp 1948), becomes an important subject of investigation because of its customary large consumption in Southeast Asian countries and the fact that the major alkaloids of betel-nut, arecoline and arecaidine, are capable of inducing cell transformation (Ashby et al. 1979); arecoline is also capable of breaking DNA strands in the bone marrow cells in vitro (Panigrahi and Rao 1982). These reports of work using purified alkaloids of the betel-nut (arecoline, arecaidine etc.) provide valuable information about their role in the process of carcinogenesis. But the results may not be the same when a complex mixture of alkaloids and other organic materials, such as those in betel-nut, is administered. This information, however, will be very relevant to the human condition where betel-nut chewers are exposed to such a complex mixture of alkaloids and other organic materials. The behaviour of a purified alkaloid and that of the same alkaloid in combination with other chemicals may not be the same. Furthermore, in a biological environment, these chemicals, especially alkaloids, are likely to be metabolized to produce an array of metabolites that may also influence the process of carcinogenesis. In addition, we have no information on damage to DNA

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Offprint requests to: R. N. Sharan at his current address: Institut für Medizin, KFA, Postfach 1913, D-5170 Jülich, FRG

caused by betel-nut products (IARC 1985). With this in mind, we have attempted to shed more light on the situation. We report the ability of aqueous extracts of betel-nut (AEBN) to break DNA strands of mouse kidney cells in vitro. We relate this event to the process of transformation.

Materials and methods

Chemicals. All chemicals for tissue culture were of tissue-culture grade. Other chemicals were of analytical grade and were used without further purification. Deionized double-distilled water was used to prepare reagents and solutions.

Preparation of betel-nut extract. For these tests, 250 g ripe betel-nuts from the West Khasi Hills area were obtained from the local market. After removal of the fibrous coats, the nuts were ground and suspended in 250 ml distilled water and kept at room temperature for 24 h. The suspension was filtered through a 0.45 μ m Millipore filter and the filtrate was lyophilized in a Secroid Lyolab BII lyophilizer. The lyophilized mass was kept at 4 °C until use. This procedure is henceforth called aqueous extract of betel-nut (AEBN).

Tissue culture. Three-week-old Swiss albino mice (male and female) were sacrificed for kidney cell culture. The kidneys were aseptically removed, minced and suspended in saline solution. After thorough washing the tissue was trypsinized at 37 °C in 0.25% trypsin solution in Ca^{2+} - and Mg^{2+} -free-phosphate-buffered saline. Sterile Eagle's minimal essential medium, buffered with sodium bicarbonate (2.8%), containing 100 units/ml antibiotics (streptomycin, penicillin and fungizone) and 10% fetal calf serum was used as growth medium. Cells (2.85×10^5) were dispensed into each culture tube and incubated at 37 ± 0.2 °C in a sterile incubator.

Enumeration of cells in culture. Enumeration of viable cells in the culture tubes was done by a dye-exclusion technique (Phillips 1973). In brief, a drop of the content of each tissue-culture tube was stained with 1% trypan blue. Five minutes later this was counted in a light microscope using a haemocytometer. From this, the population doubling time and the toxicities of AEBN or arecoline treatments were calculated by counting the viable and dead cell populations.

Treatment of cell culture with arecoline and AEBN. Sterile arecoline (1 mg/ml) and AEBN (5 mg/ml) stock solutions were prepared in Hanks' balanced salt solution. Cultures were exposed to these two chemicals on the day of seeding. A single dose of arecoline (10 μ g/ml) and two doses of AEBN (100 μ g/ml and 250 μ g/ml) were used in these experiments. A dose of 100 μ g/ml AEBN contains approximately 2%–3.5% arecoline and 2%–3% arecaidine as analysed in our laboratory, higher values than that reported in the literature (see IARC 1985).

Chromatography. The alkaline DNA-unwinding technique of Rydberg (1975) was employed with minor modifications to analyse DNA strand breaks on hydroxyapatite columns. The exact method has been described in our earlier publication (Srivastava et al. 1982). DNA estimation was carried out according to Burton (1968) with minor modifications.

Results and discussion

Figure 1 shows the viable cell growth with respect to time for control (untreated), arecoline-treated and

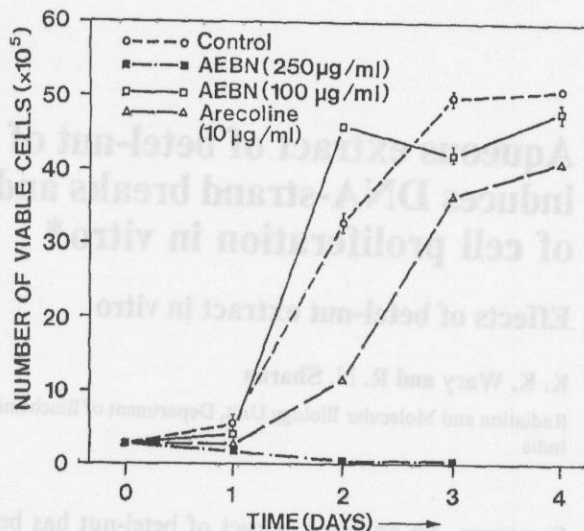


Fig. 1. Count of viable cells as a function of time for control, arecoline and AEBN-treated mouse kidney cells in culture (see also Materials and methods). Each point is mean \pm SD (shown by bars) of 5–7 independent experiments. No bar means that SD is smaller than the thickness of the point

AEBN-treated cultures. The control tubes showed approximately 100% increase in population after 18–19 h with confluent monolayer formation on the 3rd day after seeding. The arecoline (10 μ g/ml)-treated cells showed a delayed cell duplication time and monolayer formation. While 250 μ g/ml AEBN was drastically toxic to the cells, the culture tubes with 100 μ g/ml AEBN exhibited attainment of confluency a day earlier than the control. However, it showed an approximately 10% lower viable cell count. Owing to the acute toxicity of 250 μ g/ml AEBN, this dose has not been used in the following experimental protocol.

Figure 2 shows the results of DNA strand break analysis. Damage to DNA has been expressed as the ratio of ssDNA/dsDNA recovered. Arecoline-treated cells show a very significant increase in the ssDNA/dsDNA ratio (which means DNA damage) with time. The damage reduces slightly after 3rd day. The AEBN-(100 μ g/ml)-treated cells show significant damage to DNA compared with the controls, but this is not as drastic as that caused by arecoline. These data have been tabulated from the total amounts of ssDNA and dsDNA recovered from hydroxyapatite columns from different groups of cells (data not shown here).

The dose of AEBN to be administered in our experiment was determined such that we have a dose comparable to that to which the population in this area is exposed following the customary consumption of betel-nuts. On the basis of our surveys (to be communicated separately), a dose of 100 μ g/ml AEBN was found appropriate. In addition, a higher dose of 250 μ g/ml AEBN was also chosen. Similarly, the dose

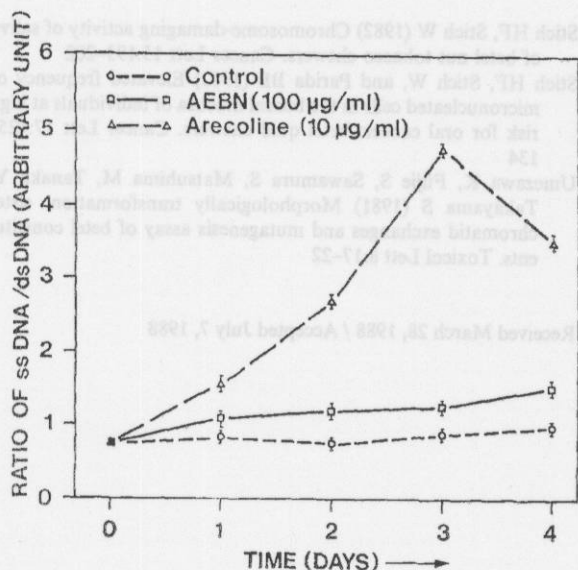


Fig. 2. Damage to DNA (expressed as the ratio of ssDNA to dsDNA) as function of time for control, arecoline and AEBN-treated mouse kidney cells in culture (see also Materials and methods and Srivastava et al. 1982). Each point is the mean \pm SD (shown by bars) of at least four determinations

of arecoline was adjusted to have approximately the same amount of this alkaloid present as the average total alkaloids in 100 μ g/ml AEBN (see IARC 1985).

We observe no morphological signs of transformation until the end of 4 days with either arecoline or AEBN, except for a reduced cell-cycle time (see below). Ashby et al. (1979) have reported induction of cell transformation by both arecoline and arecaidine from betel-nut. The difference in our results could be due to the doses of arecoline (10 μ g/ml) and AEBN (100 μ g/ml); probably both are below the threshold dose for showing morphological signs of transformation in just 4 days. However, we make a significant observation that 100 μ g/ml AEBN is able to reduce the duration of the cell cycle such that monolayer formation occurs a day earlier as compared to the control tubes (Fig. 2). It is interesting to note that while 100 μ g/ml AEBN does not cause drastic damage to DNA through strand breakage (Fig. 2) it hastens the process of monolayer formation by 1 day (which could be taken as an important sign of initiation of carcinogenesis or transformation). As against this, 10 μ g/ml arecoline does cause drastic damage to DNA through strand breakage but fails to accelerate the monolayer formation. The cells of this group, instead, show a slower rate of proliferation, as the monolayer was formed on the 4th day after seeding. Both AEBN (100 μ g/ml) and arecoline (10 μ g/ml) showed respectively 5%–7.5% and 10%–12% toxicity. Panigrahi and Rao (1982) have also shown the chromosome-

breaking ability of arecoline in bone marrow cells. Our results conform with their results, but in addition we observe an important phenomenon of higher DNA damage and a lower rate of cell proliferation after 10 μ g/ml arecoline, and relatively low DNA damage and an enhanced rate of cell proliferation after 100 μ g/ml AEBN. This, therefore, suggests that in addition to an ability to break DNA strands, the AEBN has some additional factor(s) that can initiate and/or promote cell proliferation, which could eventually lead to carcinogenic transformation. These additional factors may be present in the AEBN or may be a metabolic product of the constituents of AEBN in the biological system. Apparently, optimal (or specific?) DNA damage and the presence of this (these) additional factor(s) seem necessary to enhance the cell proliferation rate because a higher dose of AEBN (250 μ g/ml) was lethal to the cells in culture (Fig. 1). This may mean that a higher dose of AEBN may cause very severe damage or more nonspecific damage to DNA so that it leads to cell death rather than transformation. Failure of arecoline (10 μ g/ml) to enhance monolayer formation could be due to lack of additional factor(s) that may have been present in AEBN. We are in the process of analysing the components of these unprocessed betel-nuts and monitoring unscheduled DNA synthesis (UDS) under these experimental conditions.

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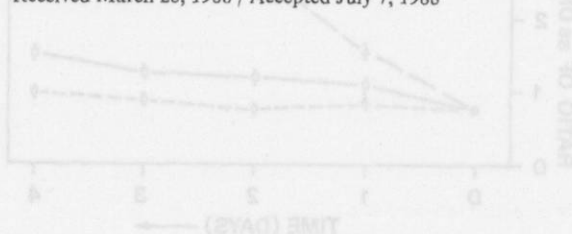


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