SPANDIDOS PUBLICATIONS

Aqueous extract of betel nut-induced adducts on pMTa4 DNA acquires stability in the presence of Na^+ and K^+ ions

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Abstract. Betel nut (BN), a natural carcinogen to humans, is used as a masticator across the globe by a large segment of the human population. The primary carcinogens of BN are alkaloids, in particular arecoline. Upon nitrosation, arecoline can potentially interact with DNA, forming adducts and initiating carcinogenesis. Though considerable evidence exists in support of the carcinogenicity of BN, the molecular mechanism of its induction of carcinogenesis is unknown. This investigation was undertaken to directly demonstrate adduct formation on DNA and to study its characteristics, such as its frequency of formation and stability. A plasmid DNA construct, pMTa4, was chosen to determine the stoichiometry and dynamics of adduct formation. This construct was exposed to aqueous extract of betel nut (AEBN) *in vitro* and *in vivo* and analyzed. Spectrophotometric analysis revealed a significant red shift in the pMTa4 DNA spectrum. The gel electrophoretic mobility of pMTa4 DNA was also retarded in an AEBN dose- and exposure time-dependent manner, indicating BN-specific adduct formation on the DNA. These results conclusively demonstrate that adducts are formed on DNA by BN extract, and suggest that one AEBN-induced adduct was formed every 3 NT on pMTa4 DNA under the experimental conditions. Trace amounts of monovalent cations, such as Na^+ or K^+ ion (≥0.5 mmol), conferred stability to the adducts on DNA, which were otherwise unstable beyond 24 h.

Introduction

Humans are constantly exposed to carcinogenic agents that interact with DNA directly or indirectly, thus inducing damage which can lead to a variety of diseases, including cancer. Betel nut (BN; *Areca catechu* L.) is one such agent, and is implicated in the induction of a wide range of cancers, including cancers of the oropharyngeal tract and hepatocarcinomas. Despite its reported association with carcinogenicity, it is estimated that BN in various forms is widely used as a masticator by over 600 million people across the globe (reviewed in refs. 1-4). In chronic chewers, it is especially implicated in oral leukoplakia and oral submucous fibrosis (OSF), and is believed to be one of the major reasons for the high incidence of oral malignancy found in Asian populations (5). Detailed physicochemical investigations have established that water-soluble alkaloids, besides some polyphenols and tannins, are the main carcinogenic component of BN (1,3). BN alkaloid consists predominantly of arecoline and arecaidine, as well as small amounts of guvacine and guvacoline, among others (1,3,4). Considerable evidence suggests that arecoline, upon nitrosation, produces at least four types of betel nut-specific nitrosamines (BSNAs) (1,3). These compounds have been isolated, purified and characterized. Being electrophilic, they have a high affinity for DNA (1,3) and consequently interact with it readily. This can produce DNA adducts (6) that potentially initiate mutagenesis, leading to carcinogenesis (7).

Arecoline and various BN extracts - in particular aqueous extract of betel nut (AEBN) - have been shown to be cytostatic and cytotoxic to the human *hep2* cell line (8), as well as being tumorigenic (9). They induce dysregulated cell cycle controls in OSF and interfere with the glutathione metabolism (10). It has been demonstrated that the metabolic activation of arecoline occurrs via nitrosation in the *hep2* cell line (8). This exposure induces strand breaks in DNA and an enhanced cell cycle (11), and prompts different types of chromosomal aberrations, including sister chromatid exchanges (10) and unscheduled DNA synthesis (UDS) (12). However, BN has not been reported to induce cellular apoptosis (13). A 2-fold induction of *c-fos* and *c-jun* protooncogene products as well as elevated *c-Myc* levels in JB6 cell lines upon exposure to betel quid suggests the induction of cellular transformation (13). These observations strongly indicate that components of BN, especially alkaloids and their activated derivatives, might form adducts on DNA. Indeed, the presence of BSNA adducts following exposure to BN or betel quid has been chemically demonstrated. Quantification of induced adducts by fluorescence-detected HPLC, gas chromatography or other biophysical methods has shown a high degree of correlation with cellular transformation (14). Thus, the involvement of adducts in BN-induced human carcinogenesis is clear. Nonetheless, to the best of our knowledge not much is known about the molecular characteristics of the DNA adducts or their consequences (3). In particular, the kinetics of BN-specific adduct formation on DNA and factors affecting their stability are totally unknown. This paper reports

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on an investigation of the molecular characterization and kinetics of AEBN-induced *in vitro* and *in vivo* formation of DNA adducts.

Our chosen model was *E. coli*, as this simple organism has been used with great success in past studies to determine the molecular characteristics of adduct formation. *E. coli* is also preferred because it often harbors or can be made to harbor plasmid DNA, which is a convenient tool for this type of study (15). Our laboratory has designed a plasmid DNA construct, pMTa4 (6173 bp), which has been successfully used to reveal the DNA damage inflicted by free radicals (16) or by different qualities of radiation (17,18). Other groups have used similar approaches (19). The plasmid pMTa4 was used in this study as well. For *in vitro* investigation, isolated plasmid DNA was exposed to AEBN in an Eppendorf tube and analyzed. For *in vivo* studies, pMTa4 was first transformed into a wild-type *E. coli* strain, AB1157. The transformed *E. coli* cells harboring pMTa4 were then exposed to AEBN under physiological conditions. Following AEBN exposure, pMTa4 was isolated from the *E. coli* cells and analyzed.

Materials and methods

Chemicals. All chemicals used were of the highest purity grade. Agarose, λ DNA *Hin*dIII digest (Genei, India), ampicillin (Duchefa, The Netherlands), ethidium bromide (EB; Merck, Germany), Tris-base (Boehringer Mannheim GmbH, Germany), ethylenediaminetetracetic acid (EDTA) and sodium dodecyl sulphate (SDS; Sigma Chemical Co., USA), KCl, HCl, acetic acid and sodium hydroxide (Qualigens, India), sodium acetate (SRL, India), NaCl, Luria Bertani (LB) broth and LB-agar (Himedia, India), and ethanol (Bengal Chemicals, India) were used.

Preparation of aqueous extract of betel nut. AEBN was prepared as previously described (20). Briefly, raw and wet varieties of BN, obtained from a local market, were dehusked, crushed to a coarse powder and soaked overnight in sterile water at room temperature. The extract was then filtered through Whatman No. 1 filter paper and lyophilized to powder. The resulting AEBN was dissolved in sterile water at appropriate concentrations (50, 100, 150, 200 and 250 μg) for the experiment.

Plasmid pMTa4 and its isolation. The previously described pMTa4 plasmid (15-18) was isolated by standard alkaline methods from an overnight mid-log culture of *E. coli* in LB broth with 100 μg/μl Ampicillin at 37˚C. The inoculum for the culture was a single colony picked up from an Ampicillin$^+$ LB-agar plate of a wild (AB1157) K12 strain of *E. coli* harboring the plasmid. The pMTa4 isolate was air dried, dissolved in sterile water and refrigerated until use.

Transformation of pMTa4 in E. coli. Transformation was performed as recently described (18). In brief, ≈75 μl (300 ng DNA) of pMTa4 was added to 200 μl of freshly prepared competent cells in a pre-cooled tube, gently mixed and kept on ice for 20 min. The tube was then incubated in sequence at 42˚C for 30 sec and on ice for 180 sec. LB medium (500 μl, pre-warmed to 37˚C) was added to the tube, gently mixed, and incubated at 37˚C for 60 min. The contents (200 μl) were then plated on LB-agar plates (with 100 μg/ml Ampicillin) at 10^{-3} and 10^{-5} dilutions, then incubated overnight at 37˚C. An LB-agar plate without ampicillin was used as the control.

Agarose gel electrophoresis. Electrophoresis was performed on a 1% agarose gel using TAE (40 mM Tris-acetate, 1 mM EDTA) running buffer at 100 V (constant) for 60 min as previously described (16,18). The gel was stained with EB (0.3 μg/ml) for 15 min, then destained in water for 30 min on a rocking plate. The EB-intercalated DNA was visualized on a UV transilluminator (Bio-Rad).

Spectrophotometric analysis of pMTa4 DNA following incubation with AEBN in vitro. pMTa4 isolates (2 μl containing 4 μg DNA) were incubated at 37˚C without or with varying concentrations of AEBN (50, 100, 150, 200 and 250 μg) for different time intervals (0, 15 and 30 min). The mixtures were diluted to 1 ml with sterile water and the absorbance spectra were recorded over a range of 235-320 nm using a spectrophotometer (Systronics, UV-Vis Spectrophotometer 119).

AEBN-induced mobility shift of pMTa4 as a function of the concentration of AEBN in vitro. pMTa4 isolates (2 μl containing 4 μg DNA) were incubated at 37˚C for 30 min without or with varying concentrations of AEBN (50, 100, 150, 200 and 250 μg). Following exposure, the samples were subjected to agarose gel electrophoresis.

AEBN-induced mobility shift of pMTa4 as a function of the time of incubation in vitro. pMTa4 isolates (2 μl containing 4 μg DNA) were incubated with 250 μg of AEBN at 37˚C for different time periods (0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 min), then subjected to agarose gel electrophoresis.

Stability of AEBN-DNA adduct in vivo. AEBN (2000 μg ml) was added to a flask containing LB medium and 100 μg/μl ampicillin. It was inoculated with a single colony of *E. coli* from an ampicillin$^+$-agar plate. The control was normal medium without AEBN. The cultures were grown overnight at 37˚C. Plasmid was then isolated from the cultures. Equimolar aliquots of pMTa4 isolate were dissolved in 30 μl of sterile water and incubated at room temperature for increasing time intervals up to 24 h. Samples were taken out every two hours for evaluation by agarose gel electrophoresis.

Effects of pH and the Na^+ and K^+ ions on the stability of AEBN-induced adducts. In order to study the effects of pH and monovalent cations on the stability of AEBN-induced pMTa4 adducts, equimolar amounts of air-dried pMTa4 DNA (isolates) were taken in different tubes. To study the effects of pH on the stability, the pMTa4 isolates were dissolved separately in 30 μl of 1 M Tris-Cl buffers at pH 5.0, 6.0, 7.0 or 8.0. The control pMTa4 DNA was dissolved in sterile water. The tubes were incubated at room temperature for 24 h, then subjected to agarose gel electrophoresis. To study the effects of monovalent cations (e.g., Na^+ and K^+) on stability, the pMTa4 isolates were dissolved separately in 30 μl sterile water containing different concentrations of either KCl (0.5, 5, 50 and 500 mmol) or NaCl (0.5, 5, 50 and 500 mmol). pMTa4

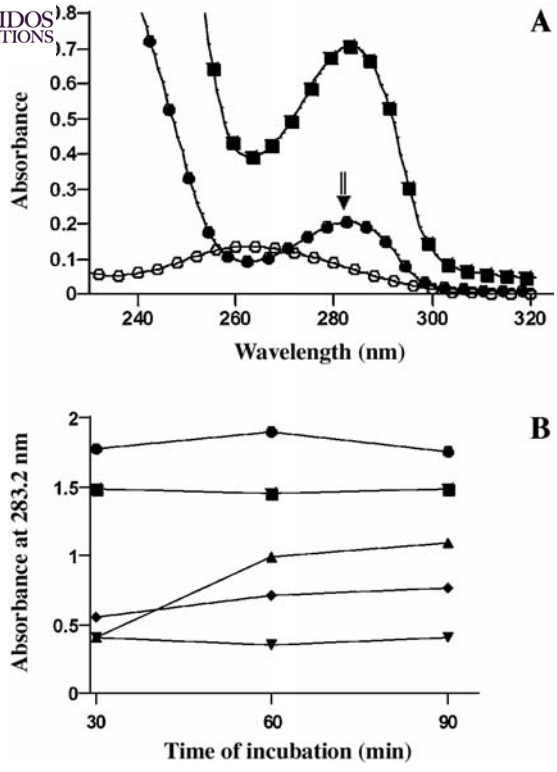

Figure 1. Spectrophotometric analyses of pMTa4 and AEBN interaction *in vitro*. (A) Absorbance spectra of pMTa4 DNA (○), AEBN (■) and pMTa4 exposed to 250 μg AEBN for 30 min at 37˚C (●). (B) Absorbance maxima (283.20 nm) of pMTa4 exposed to 50 μg (▼), 100 μg (◆), 150 μg (▲), 200 μg (■) and 250 μg (●) AEBN for increasing time periods at 37˚C.

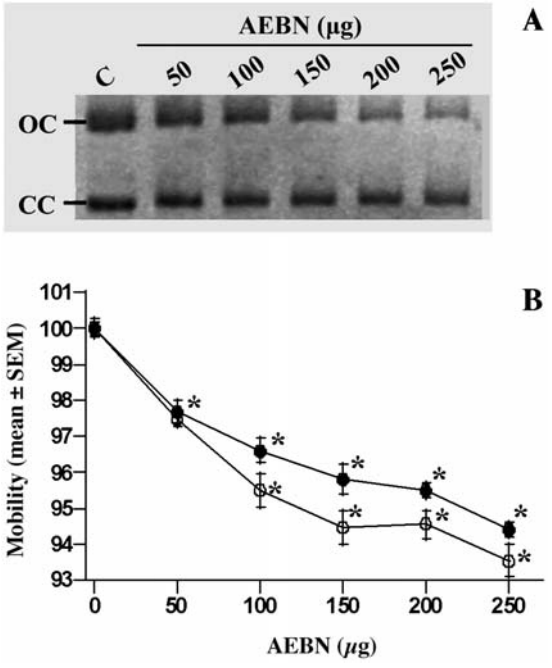

Figure 2. Effect of increasing doses of AEBN on the gel electrophoretic mobility of plasmid pMTa4 *in vitro*. (A) Retardation of the mobility of the CC and OC forms of pMTa4 with increasing doses of AEBN [lane 1 (C), unexposed pMTa4 (4 μg); lanes 2-6, pMTa4 (4 μg) exposed to 50, 100, 150, 200 and 250 μg of AEBN respectively for 30 min at 37˚C]. (B) Percentage mobility changes for the CC (●) and OC (○) topological forms of pMTa4 as a function of the increasing dose of AEBN. Data (mean ± SEM) were obtained from the electropherogram (A). *Statistically significant ($p \leq 0.01$) compared to the controls.

DNA dissolved in sterile water served as the control. The samples were incubated at room temperature for 24 h, then subjected to agarose gel electrophoresis.

AEBN-induced increase in the molecular size of the linear form of pMTa4. The plasmid (pMTa4) was linearized using a restriction endonuclease, *Nco*I, which has only one restriction site in the plasmid (16,18). Aliquots (2 μl) of the linear (L) form of pMTa4 (4 μg) were incubated without or with increasing concentrations of AEBN (50, 100, 150, 200 and 250 μg) at 37˚C for 30 min, then subjected to agarose gel electrophoresis.

Data capture, analysis and statistical evaluation. A minimum of five independent experiments were conducted for each investigation/point. The plasmid DNA bands on agarose gels were digitized (Kodak) immediately after destaining. The band intensities of different topological forms of pMTa4 were calculated as previously described (16,18). From the digitized photographs, the mobility of the bands was calculated using gel documentation and analysis software (KDS1D; Kodak). The mean ± SEM of all the data was calculated, and graphs were plotted using Kaleidagraph software. The student's t-test was applied for statistical evaluation. $p \leq 0.01$ was considered significant.

Results

The method of plasmid isolation employed by the study routinely produced ≈40 μg DNA from a culture of 9×10^9 *E. coli* cells. The plasmid isolate was dissolved in sterile water at a concentration of 2 μg/μl. The purity of the isolate was high (a ratio of 260/280, ≈1.9), and the native conformation of the plasmids condensed, covalently closed circular (CC). However, during plasmid isolation some single strand breaks were inevitably induced on the CC form and resulted in the formation of an open circle (OC) or relaxed topological form of the plasmid (16,18). The plasmid isolates used included about 60% CC and 40% OC forms. The transformation frequency of pMTa4 plasmids into *E. coli* was moderate to high according to the protocol used.

AEBN-induced spectral red shift in vitro. Fig. 1A shows the typical absorbance spectra of pMTa4, AEBN and pMTa4 exposed to 250 μg AEBN for 30 min at 37˚C. As expected, pMTa4 reached absorption maximum at 260.80 nm, while AEBN peaked at 283.20 nm. AEBN-exposed pMTa4 showed maximum absorption at 283.20 nm (arrow), recording a red spectral shift of 19.60 nm as compared to pMTa4. Fig. 1B plots the absorbance maxima (283.20 nm) of pMTa4 exposed to increasing doses of AEBN for different time periods at 37˚C. A time-dependent increase in absorbance was only observed for a dose of 150 μg AEBN.

AEBN concentration-dependent gel mobility shift of pMTa4 DNA in vitro. The gel in Fig. 2A shows CC and OC topological forms of unexposed control pMTa4 (lane 1) and pMTa4 exposed to increasing doses of AEBN (lanes 2-6). Retardation of the mobility of both the CC and OC forms is noticeable. The plot of percent mobility shift as compared to the mobility of the CC and OC form controls is shown in Fig. 2B. In a

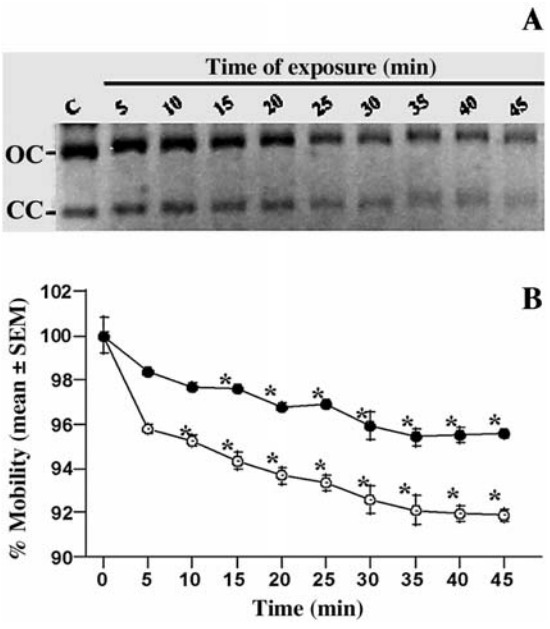

Figure 3. Effect of the duration of AEBN exposure on the gel electrophoretic mobility of plasmid pMTa4 *in vitro*. (A) Retardation of the mobility of the CC and OC forms of pMTa4 with increasing duration of exposure to AEBN [lane 1 (C), unexposed pMTa4 (4 μg); lanes 2-10, pMTa4 exposed to 250 μg of AEBN for 5, 10, 15, 20, 25, 30, 35, 40 and 45 min respectively at 37˚C]. (B) Percentage mobility changes for the CC (●) and OC (○) topological forms of pMTa4 as a function of the time of AEBN exposure. Data (mean ± SEM) were obtained from the electropherogram (A). *Statistically significant ($p \leq 0.01$) compared to the controls.

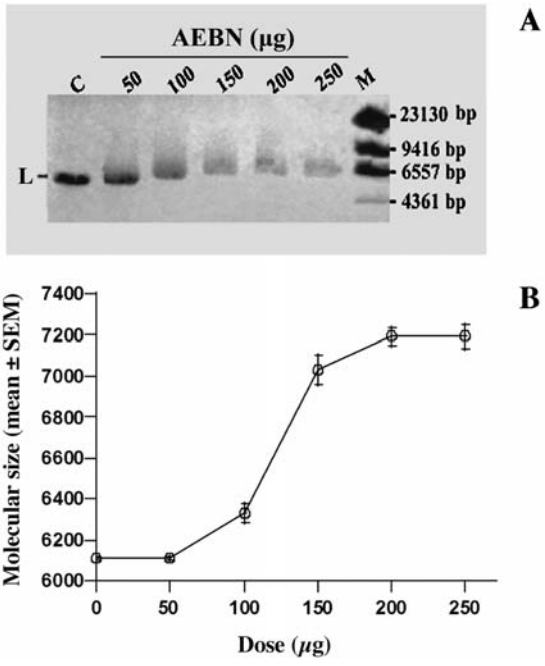

Figure 4. Increase in the molecular size of pMTa4 DNA upon exposure to AEBN. (A) Retardation of the mobility of linearized pMTa4 DNA exposed to increasing doses of AEBN [lane 1 (C), unexposed pMTa4 (4 μg); lanes 2-6, pMTa4 (4 μg) exposed to 50, 100, 150, 200 and 250 μg AEBN respectively at 37˚C for 30 min; lane 7 (M), DNA marker (λ DNA *Hin*dIII digest) in molecular size (bp)]. (B) Increase in bp of pMTa4 as a function of the dose of AEBN. Data (mean ± SEM) were obtained from the electropherogram (A).

relative sense, the OC form of pMTa4 exhibited a higher retardation than the CC form.

AEBN exposure period-dependent gel mobility shift in pMTa4 DNA in vitro. The gel in Fig. 3A shows CC and OC topological forms of unexposed control pMTa4 isolate (lane 1) and pMTa4 exposed to 250 μg of AEBN over increasing time periods (lanes 2-10). Retardation of the mobility of both the CC and OC forms is noticeable. The plot of percent mobility shift as compared to the mobility of the CC and OC form controls is shown in Fig. 3B. In a relative sense, the OC form of pMTa4 again exhibited a higher retardation than the CC form.

AEBN-induced increase in the molecular size of pMTa4. The gel in Fig. 4A shows *Nco*I linearized unexposed control pMTa4 (linear form or L; lane 1) and pMTa4 exposed to increasing doses of AEBN (lanes 2-6) for 30 min at 37˚C. An equivalent increase of ~1000 bp in the molecular size of the L form of pMTa4 was noticed at up to 150 μg AEBN, after which it remained invariant (Fig. 4B).

Kinetics of the breakdown of adduct. Fig. 5A shows a gel of CC and OC topological forms of unexposed control pMTa4 (lane 1) and aqueous solutions of pMTa4 withdrawn every two hours from AEBN exposure at room temperature (lanes 2-13). The plot of percent mobility shift as compared to the mobility of the CC and OC form controls is shown in Fig. 5B. The graph shows that the maximum retardation in the mobility of the CC and OC forms of pMTa4 occurred between 6 and 8 h. Subsequently, the trend progressively reversed. Both forms regained almost normal gel mobility by 24 h.

Effects of pH, Na^+ and K^+ on the stability of adducts. The plots of percent mobility shift as compared to the mobility of the control CC and OC forms of pMTa4 under different treatment conditions are shown in Fig. 6. The results indicate that increasing pH, in the range of pH 5.0-8.0, did not essentially cause any change in the mobility of the CC and OC forms of pMTa4 (Fig. 6A). However, the presence of even trace amounts (0.5 mmol) of either of the monovalent cations K^+ (Fig. 6B) or Na^+ (Fig. 6C) slowed down the mobility of the CC and OC forms of pMTa4 as compared to the controls. The effect was more pronounced in the OC than the CC form.

Discussion

Adduct formation is known to cause a red shift in the absorption (21) and fluorescence (22) spectra of DNA in a dose-dependent manner. Our results (Fig. 1A) reveal that the spectrum of pMTa4 DNA shifted towards pure AEBN when the plasmid was exposed to 250 μg of AEBN for 30 min at 37˚C. A significant 19.60 nm red shift of the spectrum of the mix suggests that AEBN interacted with pMTa4 DNA *in vitro* under the experimental conditions. Time as well as concentration kinetic studies were performed to determine the optimum or saturating dose and period of exposure to AEBN for such interaction. Fig. 1B shows an AEBN dose- and exposure time-dependent increase in absorption at 283.20 nm. Since only one concentration of AEBN (150 μg) per 4 μg pMTa4 DNA at 37˚C exhibited dose-dependence, we extrapolated that a concentration of AEBN above this would be saturating. Therefore, for all further *in vitro* studies, 4 μg of pMTa4 was exposed to 250 μg of AEBN for 30 min at 37˚C

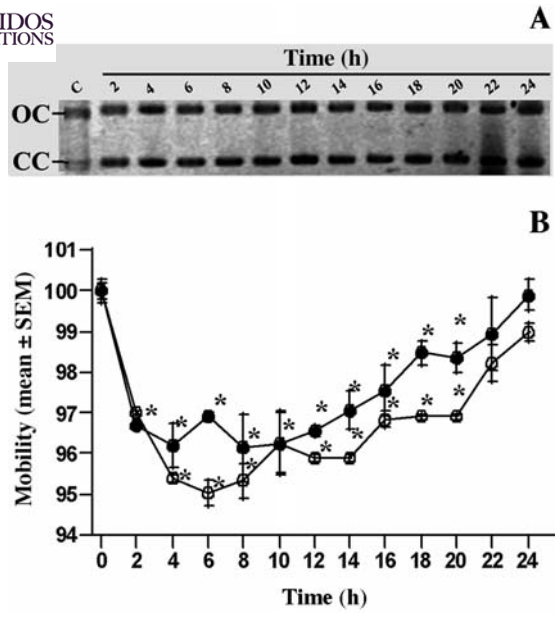

Figure 5. Time-dependent restoration of the mobility of pMTa4 upon the withdrawal of the AEBN exposure regime. (A) Change in the mobility of the CC (●) and OC (○) topological forms of pMTa4 isolated from AEBN-exposed *E. coli in vivo* as a function of the time of AEBN exposure withdrawal [lane 1 (C), unexposed pMTa4; lanes 2-13, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h after the withdrawal of the AEBN exposure regime, respectively]. (B) Kinetics of the restoration of the mobility of the CC and OC forms of pMTa4 as a function of time after AEBN exposure withdrawal. Data (mean ± SEM) were obtained from the electropherogram (A). *Statistically significant ($p \leq 0.01$) compared to the control.

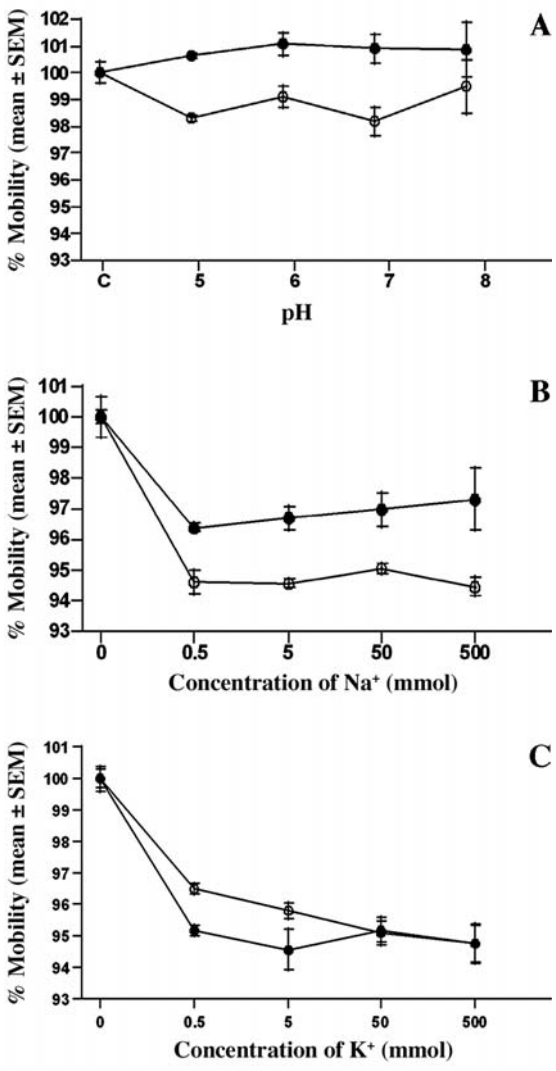

Figure 6. Effects of pH and the Na^+ and K^+ ions on the stability of AEBN-induced pMTa4 DNA adducts. (A-C) Mobilities of the CC (●) and OC (○) topological forms of pMTa4 24 h after incubation at different pH (A) or in the presence of increasing concentrations (mmol) of monovalent cations Na^+ (B) and K^+ (C). Controls were sham treated. Data (mean ± SEM) were obtained from the electropherograms. See text for further details.

in order to ensure that the maximum interaction of AEBN with pMTa4 occurred.

This interaction is likely to lead to adduct formation under experimental conditions (4). Our results show that the exposure of pMTa4 to AEBN caused statistically significant retardation of the mobility of both the CC and OC forms of the plasmid in an AEBN dose- (Fig. 2) and duration of exposure- (Fig. 3) dependent manner. The OC form of the plasmid exhibited relatively higher retardation. Considering the fact that pMTa4 exposure to AEBN led to a red spectral shift, and that the mobility of the plasmid on agarose gel was retarded, one can conclude that the cause of both these observations was the formation of adducts on pMTa4 DNA upon its exposure to AEBN. The increased size of pMTa4 DNA due to adducts would reduce its mobility on agarose gel during electrophoresis. The nitrosation of arecoline has been shown to convert it into several species of electrophilic BSNA. This readily interacts with and forms adducts on DNA via weak interactions or chemical bonds, since the DNA is highly acidic (4). Arecoline or AEBN has also been shown to be mutagenic (1,3,10). This strongly suggests that, in the experimental conditions used in the present study, AEBN-induced adducts were formed on pMTa4 DNA. The OC form of the plasmid exhibited a higher slope of curve of retardation of mobility than the CC form (Figs. 2B and 3B), suggesting that it had accumulated a higher quantum of adduct than the CC form. This is to be expected, as the OC form of plasmid is a relatively relaxed conformation (15-18), leading it to offer a higher probability of interaction with AEBN than the CC form under identical conditions. The chemical nature or identity of adducts has not been ascertained in this study. However, our observation of the formation of AEBN-induced DNA adducts (Figs. 2 and 3) is supported by reported biological observations of AEBN exposure resulting in DNA strand breaks (11), UDS (12), cell cycle alteration (8,9,11), chromosomal aberrations (10), etc.

In order to determine the quantum of adducts formed on pMTa4 DNA *in vitro* by exposure to AEBN under saturating conditions, it was necessary to determine the precise change in the molecular size of pMTa4. As plasmid DNA is CC DNA, its migration on gel during electrophoresis is influenced by its net charge as well as its conformation. Consequently, the CC and OC forms of the plasmid, despite being identical in molecular size, show distinctly different migrations on gels (Figs. 2A, 3A and 4A). To quantify the increase in molecular size of pMTa4 DNA by AEBN exposure, it was therefore necessary for the plasmid to be initially linearized and then exposed to increasing doses of AEBN. The migration of the L form of plasmid DNA on gel during electrophoresis was considered to be directly proportional to its size. Fig. 4 shows

that, with an increasing dose, the mobility of the L form of pMTa4 decreased progressively, reaching a maximum at 150 μg of AEBN and remaining at that level for higher doses (Fig. 4B). The total increase in the molecular size of pMTa4 DNA was equivalent to 1000 bp under the experimental conditions. The average molecular weights of nucleotides (NT) and BSNA are 283.25 and 126.6 Da, respectively. A shift of approximately 1000 bp (corresponding to an increase of 2000 NT in two strands of DNA) was seen to occur in the L form of pMTa4 DNA. Hence, it follows that for a 1000 bp increase in the molecular size, ≈1100 adducts should have been formed on a piece of double-stranded DNA. Based on the results and the fact that pMTa4 DNA is 6173 bp in size, it can be hypothesised that AEBN-induced adduct formation occurred every 3 NT under the experimental conditions.

To study the stability of the adducts on DNA, *E. coli* harboring pMTa4 was exposed to 2000 μg/μl of AEBN - a dose equivalent to the saturating dose of AEBN *in vitro* - under *in vivo* conditions in separate experiments. The isolated plasmid DNA was re-established in aqueous solution every 2 h for up to 24 h, and then subjected to electrophoresis to determine the persisting extent of the retardation of mobility of the OC and CC forms. The result shows that retardation of mobility reached a maximum between 6 and 8 h (Fig. 5B; 6 h in the case of the OC form or 8 h in the case of the CC form). The trend then reversed and the OC and CC forms regained their normal mobilities in about 24 h (Fig. 5). As retardation in the mobility of a DNA band was due to adduct formation, progressive reversal indicates a loss of adducts from the DNA. This demonstrates that, after an aqueous solution of AEBN-exposed pMTa4 was incubated for 6-8 h at room temperature, the adduct progressively began to dissociate from pMTa4 DNA. All adducts were lost by 24 h, and the CC or OC forms of pMTa4 DNA, now devoid of any adduct, exhibited normal mobility on agarose gel. Therefore, the nature of AEBN-induced adducts on DNA appears to be unstable under the experimental conditions. There are many possible reasons for the observed loss of AEBN-induced adducts from pMTa4 DNA. The bonding between adduct and DNA could be non-covalent in nature, or could be based on weak interactions. Both such bonds could easily be broken. Alternately, as the pH of AEBN was acidic (≈5), adducts could undergo acid hydrolysis, or other natural components of BN included in the AEBN could contribute to the observed dissociation. This aspect shall be the subject of further investigation. However, in this study we conducted experiments to find the conditions which affect the observed instability of AEBN-induced adducts. pH in the range of 5.0-8.0 did not alter the instability of the adducts on either the CC or OC forms of pMTa4, as their mobility remained invariant (Fig. 6A). On the other hand, the presence of even trace amounts of monovalent cations conferred significant stability to the AEBN-induced pMTa4 DNA adducts (Fig. 6B and C). Due to biological relevance, two such cations, namely Na^+ and K^+, were tested in this investigation. The results show that the presence of even trace amounts (0.5 mmol) of Na^+ (Fig. 6B) or K^+ (Fig. 6C) conferred significant stability to the adducts. Since intracellular concentrations of Na^+ or K^+ in mammalian cells are in the range of 10 and 140 mmol respectively (23), the results suggest that BN-specific adducts on genomic DNA are likely to remain stable under normal metabolic conditions. The stability or continuity of adducts on genomic DNA may potentially create conditions for the induction and fixation of mutation, thereby strengthening the link between BN and the etiology of human carcinogenesis.

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References

1. IARC: Tobacco habits other than smoking: betel quid and areca nut chewing and some related nitrosamines. In: Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37. International Agency for Research on Cancer, Lyon, pp141-200, 1985.
2. IARC: Betel quid and areca nut chewing. In: Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 85. International Agency for Research on Cancer, Lyon, p39, 2004.
3. Sharan RN: Association of betel nut with carcinogenesis - a review. Cancer J 9: 13-19, 1996.
4. Chiba I: Prevention of betel quid chewer's oral cancer in the Asian-Pacific area. Asian Pac J Cancer Prev 2: 263-269, 2001.
5. Trivedy CR, Craig G and Warnakulasuriya S: The oral health consequences of chewing areca nut. Addict Biol 7: 115-125, 2002.
6. Péréz-Cabré M, Cervantes G, Moreno V, Prieto MJ, Pérez JM, Font-Bardia M and Solans X: Pd(II) and Pt(II) complexes with aromatic diamines: study of their interaction with DNA. J Inorg Chem 98: 510-521, 2004.
7. Gaskell M: Comparison of the mutagenic activity of the benzene metabolites, hydroquinone and para-benzoquinone in the *supF* forward mutation assay: a role for minor DNA adducts formed from hydroquinone in benzene mutagenicity. Mutat Res 554: 387-398, 2004.
8. Wary KK and Sharan RN: Cytotoxic and cytostatic effects of arecoline and sodium nitrite on human cells *in vitro*. Int J Cancer 47: 396-400, 1991.
9. Chang MC, Ho YS, Lee PH, Chan CP, Lee JJ, Hahn LJ, Wang YJ and Jeng JH: Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. Carcinogenesis 22: 1527-1535, 2001.
10. Kumpawat K, Deb S, Roy A and Chatterjee A: Genotoxic effects of raw betel-nut extract in relation to endogenous glutathione levels and its mechanism of action in mammalian cells. Mutat Res 538: 1-12, 2003.
11. Wary KK and Sharan RN: Aqueous extract of betel-nut of North-East India induces DNA strand breaks and enhances cell proliferation *in vitro*. J Cancer Res Clin Oncol 114: 579-582, 1988.
12. Sharan RN and Wary KK: Study of unscheduled DNA synthesis following exposure of human cells to arecoline and extracts of betel-nut *in vitro*. Mutat Res 278: 271-276, 1992.
13. Lin M-H, Wang C-J, Huang H-P, Chou M-Y and Chou F-P: The tumorigenic characteristics of lime-Piper betel quid-transformed JB6 cells. Arch Toxicol 78: 167-173, 2004.
14. Bartsch H, Rojas M, Nair U, Nair J and Alexandrov K: Genetic cancer susceptibility and DNA adducts: studies in smokers, tobacco chewers and coke oven workers. Cancer Detect Prev 23: 445-453, 1999.
15. Humtsoe JO, Schroeder CH and Sharan RN: Is there a relationship between nucleotide sequence and radiation induced DNA damage? In: Trends in Radiation and Cancer Biology. International Co-operation Bilateral Seminars series, vol. 29. Sharan RN (ed). Forschungszentrum Jülich GmbH, Jülich, pp29-32, 1998.
16. Odyuo MM and Sharan RN: Differential DNA strand breaking abilities of °OH and ROS generating radiomimetic chemicals and γ-rays: Study of plasmid, pMTa4, *in vitro*. Free Rad Res 39: 499-505, 2005.
17. Humtsoe JO, Schneeweiss FHA, Srivastava A, Sarma and Sharan RN: Biological effects induced by swift heavy ions of Lithium on aqueous solution of plasmid pMTa4. Radiat Effect Defect Solid 158: 603-607, 2003.

SPANDIDOS PUBLICATIONS charjee C and Sharan RN: UV-C radiation induced mational relaxation of pMTa4 DNA in *Escherichia coli* may be the cause of single strand breaks. Int J Radiat Biol 81: 919-927, 2005.
19. Budiman ME, Alexander RW and Bierbach U: Unique base-step recognition by a platinum-acrodinylthiourea conjugate leads to a DNA damage profile complementary to that of the anticancer drug cisplatin. Biochem 43: 8560-8567, 2004.
20. Pariat T and Sharan RN: Qualitative changes in mice liver HMG proteins after low dose chronic administration of aqueous extract of betel nut and diethylnitrosamine. Hepatol Res 12: 177-185, 1988.
21. Geacintov NE, Cosman M, Mao B, Alfano A, Ibanez V and Harvey RG: Spectroscopic characteristics and site I/site II classification of *cis* and *trans* benzo(a)pyrene diolepoxide enantiomer-guanosine adducts in oligonucleotides and polynucleotides. Carcinogen 12: 2099-2108, 1991.
22. Jiang G, Jankowiak R, Grubor N, Banasiewicz M, Small GJ, Skorvaga, M, van Houten B and States JC: Supercoiling DNA promotes formation of intercalated *cis*-N_2-deoxyguanine adduct and base-stacked trans-N2-deoxyguanine adduct by (+)-7R,8S-dihydrodiol-9S,10R-epoxy-7,8,9,10-tetra-hydrobenzo(a)pyrene. Chem Res Toxicol 17: 330-339, 2004.
23. Murray RK, Granner DK, Mayes PA and Rodwell VW. In: Harper's Illustrated Biochemistry, 26th edition. McGraw-Hill Professional, pp416, 2003.