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# Qualitative change in mice liver HMG proteins after low dose chronic administration of aqueous extract of betel nut and diethylnitrosamine

Theisara Pariat, Rajeshwar N. Sharan *

*Radiation and Molecular Biology Unit, Department of Biochemistry, North-Eastern Hill University, Shillong 793 022, India*

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**Abstract**

High mobility group (HMG) proteins have roles in the organization and function of chromatin. Their involvement in carcinogenesis is, however, not clear. Swiss albino mice were exposed to aqueous extract of betel nut (AEBN) and a hepatocarcinogen, diethylnitrosamine (DEN), in a chronic low dose protocol for up to 4 weeks. Studies of their effects on the liver HMG proteins during the process showed that major HMG proteins 1, 2 14 and 17 were qualitatively affected. HMG proteins of the treated animals were found to elute earlier from CM-sephadex column than that of the control. Conformational changes in the HMG proteins under the influence of these carcinogens were observed. The results obtained in this investigation indicate that both carcinogens induced similar changes in HMG proteins of liver of mice. 



*Keywords:* Carcinogenesis; Betel nut; Diethylnitrosamine; Conformational change; Liver; Hepatocarcinogen; In vivo

* Corresponding author. Tel.: +91 364 250107 ext. 300/311; fax: +91 364 250108/250076.

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## 1. Introduction

HMG (high mobility group) proteins are basic non-histone chromosomal proteins [1] which have progressively become an important class of proteins with apparent roles in organization and functions of chromatin [2,3]. They have been shown to have critical roles in induction and regulation of gene expression and in DNA replication [1,2,4]. The HMG 1 and 2 proteins appear to be involved with DNA replication and may function as a transcription factor in actively transcribing genes [5,6]. There are indications that HMG 14 and 17 proteins may be associated with transcriptionally active genes and have regulatory functions [1,2]. Nuclei of transformed cells have, in addition to these major HMG proteins, high levels of three other HMG I proteins, which are absent or at very low levels, in terminally differentiated, non-proliferating tissues [7,8]. A possibility that the HMG I proteins, present in transformed cells, might replace HMG 14 and 17 altering gene expression either quantitatively or qualitatively has been suggested [7,9]. However, understanding of HMG I proteins remains fragmentary to extend further studies. The understanding of the major HMG proteins namely, HMG 1, 2, 14 and 17, is relatively better. Nonetheless, their roles in transformation and carcinogenesis processes remains unclear.

Consumption of betel nut (*Areca catechue* L.) by a large human population is prevalent in different parts of the world. Betel nut consumption is very strongly suspected to be associated with carcinogenesis [10]. In the north-eastern part of India, unprocessed betel nut along with betel leaf and slaked lime is consumed [11]. It has been shown that the unprocessed betel nut contained higher quantity of alkaloids [11]. Aqueous extract of betel nut caused DNA strand breaks, induced enhanced cell proliferation [12] and affected unscheduled DNA synthesis [13]. Arecoline, the main alkaloid of betel nut, has been shown to inhibit DNA and protein synthesis [14] and influence chromosomal organization [15], thereby, suggesting that arecoline influenced gene expression in mice in vivo. To probe it further, this study was designed to monitor the effects of aqueous extract of betel nut (AEBN) on mice HMG proteins. Liver being a major portal system and a pre-mitotically fixed tissue, was chosen to be suitable for the investigation. Another known hepatocarcinogen, diethylnitrosamine (DEN), which has been shown to influence gene expression in vivo [16], was used in this investigation as a positive control.

## 2. Materials and methods

### 2.1. Chemicals and animals

All chemicals used were of analytical grade or of highest purity grade available. Biochemicals were obtained from Sigma. De-ionized double distilled water was used to prepare reagents and solutions. Six to eight week-old, female Swiss albino mice (Balb/c strain) were used. They were housed in polycarbonate cages with husk

bedding in a well-ventilated animal room maintained at 25°C. Standard mouse pellet and drinking water were provided ad libitum.

### 2.2. Exposure protocol

Aqueous extract of betel nut, prepared as described earlier [13], was administered at a concentration of 0.5 mg/ml of drinking water. Similarly, diethylnitrosamine (DEN) solution of 0.0167 mg/ml was also prepared in drinking water. AEBN or DEN was chronically administered in drinking water ad libitum on four groups of mice (average body weight of mice = 25 g). In this protocol, the average dose delivered to each mouse was 300 mg/kg body weight for AEBN and 10 mg/kg body weight for DEN. The treatment was continued for 1, 2, 3 or 4 weeks. Age-matched control mice received normal drinking water. Mice were sacrificed by cervical dislocation on the day following the last day of treatment and their livers were removed. Experiments were repeated at least three times for each set.

### 2.3. Preparation of high mobility group (HMG) proteins

The procedure has been described earlier [17]. Briefly, the livers were homogenized in 5% cold perchloric acid (PCA) to make a 0.6% homogenate, left in ice for 20 min, centrifuged at 5800 × *g* for 15 min and the supernatant stored. Starting with the pellet, the extraction was repeated two more times. All the supernatants were pooled and filtered through Whatman no.1 filter paper. To the filtrate, calculated volume of HCl was added to make 0.4 N solution. Acetone (3.5 × volume) was added to the solution, mixed and immediately centrifuged at 4800 × *g* for 15 min. To the supernatant, 2.5 × volume of acetone was further added, left at room temperature for 60 min and centrifuged at 21000 × *g* for 60 min. The pellet obtained was designated as HMG fraction. The content of protein was estimated by the method of Bradford [18] using BSA as a standard.

### 2.4. Fractionation of HMG proteins

The basic procedure of Nicolas and Goodwin [19] was used with some modifications for fractionation of the HMG proteins. First, 150 μl of HMG fraction was loaded onto a CM-sephadex column and washed with equilibration buffer to remove unabsorbed proteins. Following this, a continuous gradient of NaCl (0–1.2 M) in sodium borate-mercaptoethanol buffer (pH 8.8) was applied. The absorption profile of the eluted fractions was recorded at 230 nm.

### 2.5. Spectrofluorimetric analysis

The technique described by Cantor and Schimmel [20] was essentially followed to monitor the extrinsic fluorescence of HMG proteins using a dye, 8-anilino-naphthalene-sulfonic acid (ANS). The excitation maximum of ANS was determined to be 354 nm. Fluorescence spectrum (range 400–600 nm) of distilled water alone as

well as that of aqueous solution of ANS (0.0125 μg/ml) was recorded after exciting the sample at 354 nm. Under identical conditions of excitation and emission, fluorescence spectra of HMG fractions (0.72 μg/ml) from different experimental groups were recorded.

### 2.6. Calculation of conformational characteristics

Using the fluorescence spectra, the following were calculated to evaluate conformational changes in HMG proteins: (a) area of the peak, (b) height of the peak, (c) width at half peak and (d) peak shift. For this, a constant baseline was determined on the plots. The area of peak, the width at 50% height of the peak and the height of the peak were calculated for control as well as for AEBN and DEN treated groups. The ratios of these for HMG proteins to that obtained for water (HMG peak/$H_2O$ peak) were calculated and plotted.

## 3. Results

On the whole, there was no distinguishable abnormality in the external appearance of the exposed mice. No significant change was observed in the weights of the livers of AEBN treated mice. However, there was a non significant tendency of decrease in the liver weight in the case of DEN-treated mice (Fig. 1). The HMG protein content of liver of both AEBN and DEN-treated groups showed a tendency to decline towards the control level (Fig. 2) after an initial rise in 1 week (DEN-treated) or 2 weeks (AEBN-treated).

Fig. 3 shows the profiles of eluted HMG proteins from CM-sephadex column for the untreated control and those after 1–4 weeks of AEBN or DEN exposure. A marked difference in the profile of treated groups was the absence of peak

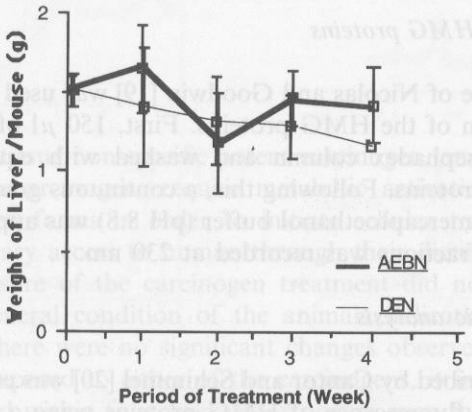


Fig. 1. Weight of liver (mean ± S.D.) of untreated mice and that of mice chronically exposed to AEBN or DEN in drinking water.

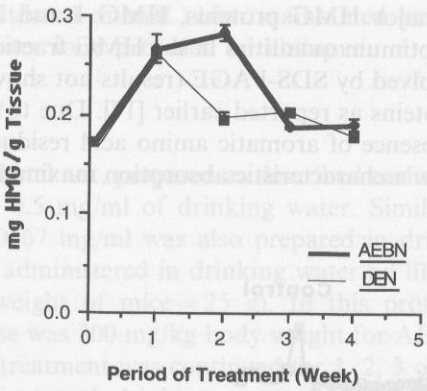


Fig. 2. The contents of total HMG proteins (mean ± S.D.) of livers of unexposed mice and that of AEBN or DEN-exposed mice. No error bar means deviation was smaller than the thickness of the point.

corresponding to HMG 1 and 2 (between fractions 10 and 20) in all treatment groups as compared to the normal pattern. The HMG 14 and 17 protein, which eluted around fractions 40–60 in the control, were advanced in all treatment groups. The 4-week group exhibited fragmentation of HMG proteins in AEBN or DEN-treated group.

Fig. 4 shows the ratios of area, height and width at 50% peak height for HMG proteins to that obtained for water (Protein/$H_2O$). The values were calculated from the extrinsic fluorescence spectra (not shown; see Sections 2.5 and 2.6). The relative width at 50% height was reduced, relative area had a tendency to rise and the relative height was invariant after exposure to AEBN. The DEN-treated groups exhibited similar trend with the exception of noticeable increase in relative area. No noticeable peak shift was recorded in both the treated groups as compared to the control.

## 4. Discussion

Both AEBN, a tissue non-specific potent carcinogen, and DEN, a tissue specific and potent hepatocarcinogen, require metabolic activation to get converted to ultimate carcinogen forms in order to initiate cellular transformation and these carcinogens find easy access to human through their dietary intakes [11,14,16,21]. The chronic exposure of the carcinogen treatment did not cause any significant changes in the general condition of the animals indicating that the doses were non-toxic. Since there were no significant changes observed in the weight of liver (Fig. 1) of mice exposed to either of the carcinogens, it indicates that cancer was not fully developed within four weeks of exposure even though it must have been initiated; the dose of AEBN or DEN, used in this study, is known to cause cancer in mice [10,11,16].

Both subclasses of major HMG proteins, HMG 1 and 2 and 14 and 17, were isolated from liver in optimum quantities in the HMG fraction by the method used. The HMG fraction resolved by SDS-PAGE (results not shown) confirmed recovery of the major HMG proteins as reported earlier [17]. Due to the presence of proline and nearly complete absence of aromatic amino acid residues in HMG 14 and 17, the HMG proteins show a characteristic absorption maximum between 220 and 230

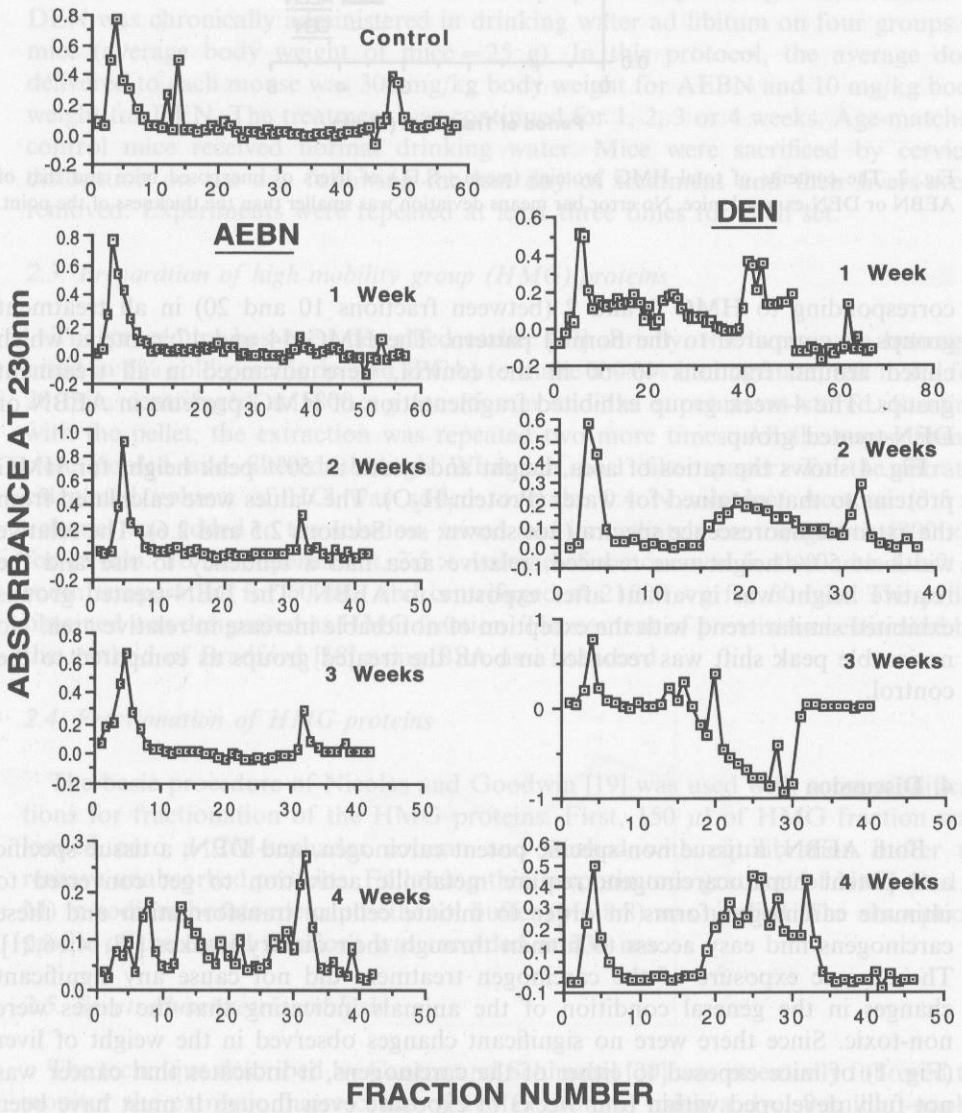


Fig. 3. Elution profile of liver HMG proteins of untreated control mice, AEBN (left panel) and DEN (right panel) from CM-sephadex column.

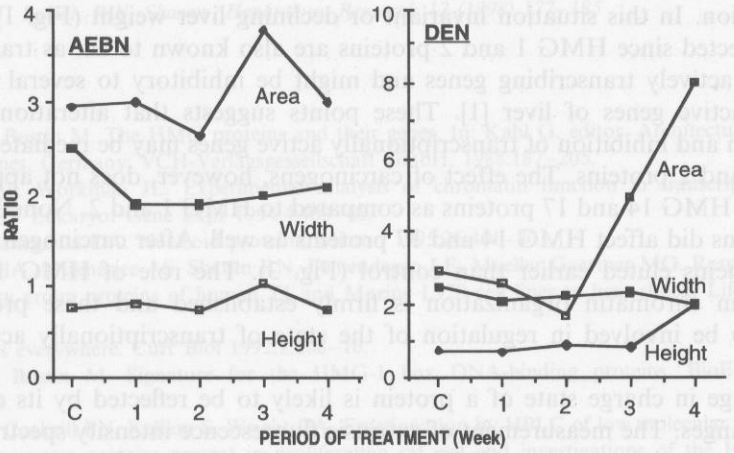


Fig. 4. Relative (arbitrary unit) area, width at half peak and height of fluorescence peak of liver HMG proteins to that of water in control, AEBN and DEN-exposed mice.

nm [19]. The HMG fraction isolated here did absorb maximally at 230 nm (result not shown), indicating that the fraction contains maximum HMG proteins. The content of major HMG proteins increased significantly upon exposure to carcinogens and declined afterwards (Fig. 2) indicating that HMG proteins were effected. The initial increase in the quantity of total HMG protein could have been due to the expression of the HMG I proteins after carcinogen exposure [22]. This was, however, not ascertained.

Since CM-sephadex column is a cation exchanger, elution of HMG 1 and 2 proteins (fraction number 10–30) and of HMG 14 and 17 proteins (fraction number 40–64) depends on the charge state of the proteins. Fig. 3 shows that the elution characteristics of these proteins changed following treatments with AEBN or DEN indicating that there must have been changes in the charge state of HMG proteins during this period. The extraction of HMG proteins by PCA is known not to effect biological activities of HMG proteins [4,19], thereby, suggesting that the isolation procedure did not cause any change. Thus, the difference in the elution of HMG proteins between control and treated groups (Fig. 3) might be due to carcinogen-induced changes in the charge states of the proteins. In contrast to the control, HMG 1 and 2 proteins after exposure to AEBN or DEN were conspicuous by their absence (Fig. 3). It is interesting that within such a short time period following low dose chronic exposure of AEBN or DEN to mice the HMG 1 and 2 proteins are effected. The absence of the peak corresponding to HMG 1 and 2 proteins, as seen in the fourth week of carcinogen exposure (Fig. 3), may also be due to carcinogen induced fragmentation which is common to HMG proteins [19]. HMG 1 and 2 proteins, particularly HMG 2, has been shown to be essential for DNA replication and consequently, affect the rate of cell proliferation [6]. Their absence or biological inactivity in very early stages of exposure to carcinogens like AEBN or DEN indicate that during initiation process there is slowing down of cell

proliferation. In this situation invariant or declining liver weight (Fig. 1) may not be unexpected since HMG 1 and 2 proteins are also known to act as transcription factor in actively transcribing genes and might be inhibitory to several transcriptionally active genes of liver [1]. These points suggests that alterations of gene expression and inhibition of transcriptionally active genes may be mediated through HMG 1 and 2 proteins. The effect of carcinogens, however, does not appear to be severe on HMG 14 and 17 proteins as compared to HMG 1 and 2. Nonetheless, the carcinogens did affect HMG 14 and 17 proteins as well. After carcinogen exposure, these proteins eluted earlier than control (Fig. 3). The role of HMG 14 and 17 proteins in chromatin organization is firmly established and these proteins are known to be involved in regulation of the state of transcriptionally active genes [1,2,23].

A change in charge state of a protein is likely to be reflected by its conformational changes. The measurement of extrinsic fluorescence intensity spectrum of the HMG proteins shows that HMG proteins underwent conformational changes following exposure to AEBN or DEN (Fig. 4). As described in Sections 2.5 and 2.6, from the extrinsic fluorescence spectra of HMG proteins of control and treated groups and water, relative area, height and width of fluorescence peak were calculated. The relative area of the fluorescence peak of HMG proteins increased significantly after 2 weeks of treatment of AEBN or DEN while the relative height of the peak as well as the relative width at half height of the peak remained essentially invariant (Fig. 4). The increased relative area is indicative of shift of molecular spin at higher level [20]. The resulting higher degree of dipole moment may lead to random orientation and distortion of superstructure of HMG proteins. In this random conformation more acidic amino acid residues may be exposed which could be the reason of advanced elution of HMG 14 and 17 proteins (Fig. 3).

Therefore, one effect of the carcinogens during initiation of carcinogenesis is changing the molecular conformation of the HMG proteins. The degrees of such changes, however, were variable for the two subclasses of HMG proteins and the two carcinogens. It is interesting that AEBN and DEN being chemical carcinogens with distinct chemical properties affected almost similar changes in liver HMG proteins. This suggests that despite being different chemical entities the two carcinogens follow similar molecular pathway during initiation of carcinogenesis in vivo. The molecular pathway, however, was not identical for the two carcinogens as evident by results presented in Figs. 3 and 4. This is expected since AEBN is a tissue non-specific carcinogen while DEN is a hepatocarcinogen. The incidence of liver cancer is higher after DEN exposure as compared to AEBN exposure [10,11,16].

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