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The usefulness of cytogenetic parameters, level of p53 protein and endogenous glutathione as intermediate end-points in raw betel-nut genotoxicity

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Betel-nut (BN) chewing related oral mucosal lesions are potential hazards to a large population worldwide. Genotoxicity of betel alkaloids, polyphenol and tannin fractions have been reported. It has been shown earlier that BN ingredients altered the level of endogenous glutathione (GSH) which could modulate the host susceptibility to the action of other chemical carcinogens. The north-east Indian variety of BN, locally known as 'kwai', is raw, wet and consumed unprocessed with betel-leaf and slaked lime and contains higher alkaloids, polyphenol and tannins as compared to the dried one. Therefore, the purpose of this study was to investigate the extent of DNA damage, pattern of cell kinetics, the level of p53-protein and endogenous GSH in kwai chewers in the tribal population of Meghalaya state in the north-eastern region of India with an aim to see whether these end-points could serve as biomarkers of genetic damage of relevance for genotoxic/carcinogenic process. The present data show higher DNA damage, delay in cell kinetics, p53 expression and lower GSH-level in heavy chewers (HC) than nonchewers (NC). The influence of bleomycin (BLM) on chromatid break induction in G_2-phase of peripheral blood lymphocytes in NC and HC has been analysed to determine individual susceptibility to carcinogenic assaults. HC showed higher induction of chromatid breaks than NC. Risk assessment in this study suggests an interaction between carcinogen exposure and mutagen sensitivity measures, risk estimates being higher in those individuals who both consume kwai and express sensitivity to free radical oxygen damage *in vitro*. From this study it seems that besides cytogenetical parameters, the level of endogenous GSH and the level of p53 protein could act as effective biomarkers for kwai chewers. *Human & Experimental Toxicology* (2003) **22,** *363–371*

Key words: biomarkers; cell cycle delay; chromosome aberrations; mutagen sensitivity; sister chromatid exchanges

Introduction

Oral squamous cell carcinoma (SCC) is the most common malignancy in India and there are strong indications for a causal association between betel-nut (BN) or quid chewing habits and oral mucosal diseases such as leukoplakia, oral submucosus fibriosis and oral cancer.[1,2] The chemical composition and pharmacological actions of BN have been reported and reviewed by several workers.[3,4] Genotoxicity of BN-alkaloids, polyphenol and tannin fractions have been reported.[4–6] BN extract is mutagenic in Chinese hamster V79 cells[7] and can induce cancerous lesions on the hamster cheek pouch[8] and also in the stomach.[9] The subcutaneous injection of BN extract leads to transplantable fibrosarcoma at the site of injection for 60% of Swiss mice involved.[10]

Reduced GSH, a tripeptide containing cysteine, is an important thiol compound present in cells. It plays an important role in regulation of cellular proliferation and cellular defense against radiation[11,12] and various xenobiotics[13,14] but not against radiomimetic drugs like bleomycin (BLM).[15,16] The exposure of buccal keratinocytes to BN and arecoline (ARC), an alkaloid of BN, leads to GSH depletion,[17] with no concomitant rise in GSH-disulfide levels, suggesting that the toxicity of BN is possibly not mediated directly by reactive oxygen species (ROS). It was shown that ARC induced chromosomal aberrations (CA) in mice bone marrow cells was enhanced by buthionine sulfoximine (BSO), a GSH-synthesis inhibitor[6] and reduced when ARC admi-

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nistered with N-acetyl-L-cysteine.[18] Therefore, the alteration in the level of endogenous GSH by BN ingredients will modulate the host susceptibility to the action of other chemical carcinogens.

Mutation in the p53 gene, a tumour suppressor gene, is one of the most common genetic abnormalities found in human cancers, especially in the development of head and neck cancers.[19,20] Elevated levels of p53 protein have been observed not only in oral SCC but also in oral dysplastic lesions, suggesting that p53 alteration is an early event in oral carcinogenesis.[21] These findings suggest that inactivation of p53 protein may precede over tumour development in oral tumorigenesis and thus it may serve as an intermediate biomarker for risk assessment.

The north-east Indian variety of BN is raw, wet and consumed unprocessed with betel-leaf and slaked lime. The constituents of this nut show higher alkaloids, polyphenol and tannins compared to the dried one[22] and is locally known as 'kwai'. Stich *et al.*[23] demonstrated the genotoxic potentiality of saliva of kwai-chewers of the tribal population of Meghalaya state of the north-eastern region of India in Chinese hamster ovary cells. The average age of onset of chewing among tribes was about 12 years, and at 35 years of age and older, the frequency of oral carcinoma rose significantly. Therefore, the purpose of this study was to investigate the extent of DNA damage, the pattern of cell kinetics, the expression of p53 protein and endogenous GSH level in kwai chewers in the tribal population of Meghalaya state of the north-eastern region of India with an aim to see whether these end-points could serve as biomarkers of genetic damage of relevance for carcinogenic process. Moreover, an attempt has been made for mutagen sensitivity assay of these chewers since such assay may reflect either an underlying DNA repair deficiency or factors which control susceptibility to initial clastogenic influences.[24]

Methods

Chemicals

Ficoll-hypaque, 5-bromodeoxyuridine, Nonidet P-40, sodium dodecylsulphate, aprotinin, 5,5'-dithiobis (2-nitro benzoic acid) (DTNB), NADPH and GSH-reductase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33258 was obtained from Roche Chemicals (Mannheim, Germany). Giemsa stain was obtained from BDH Chemicals Ltd (Poole, UK) and BLM sulphate from Biochem Pharmaceutical Industries (Mumbai, India). Other chemicals used in this study were of analytical grade.

Selection of subjects

Peripheral blood was collected from 35 donors in heparinized vials, under aseptic condition. Of 35 donors, 25 were chewers of a mixture of kwai, lime and betel-leaf, (hereafter referred to as chewers) and 10 healthy non-chewing (NC) individuals as controls. Among the 10 NC controls, five were male. The 25 chewers, mostly male, were further classified as a) moderate chewers (MC) consuming around 40–55 g of nut with an average 20 chews per day and b) heavy chewers (HC) consuming around 100–120 g of nut with an average 40 chews per day. Most of these donors were nonsmokers except two or three in each category with a mild smoking habit. All the donors had had no viral disease or antibiotic therapy during the last 6 months. Informed consent was obtained from all the individuals studied before sample collection.

Blood collection and culture were done in two batches. From these collected samples 7 NC (control) and 5 HC were selected randomly for mutagen sensitivity assay. In another set 8 NC and 8 HC were randomly selected for the determination of the level of p53 protein. The endogenous total-GSH was estimated from each sample. The controls and the chewers were preidentified from the staff members of our university and nearby villages.

Culture procedure

Heparinized peripheral blood (1 mL) was added to 7.0 mL Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) supplemented with 15% heat inactivated fetal calf serum (Biological Industries Ltd., Israel) following phytohaemagglutinin (PHA; Gibco, USA) stimulation and incubated at 37°C. To obtain differential sister chromatid staining, 5 μg/mL 5-bromodeoxyuridine was added to the cultures at the time of initiation. Cells were harvested at 72 hours and colcemid (0.01 mg/mL) was added 3 hours prior to that.

For mutagen sensitivity assay the standard blood lymphocyte cultures were set up as it was mentioned above without 5-bromodeoxyuridine. At 67 hours of incubation, cultures were treated with BLM 30 μg/mL for 5 hours. Colcemid (0.02 mg/mL) was added in the last 2 hours to induce mitotic arrest prior to harvesting.

Preparation of metaphases

Conventional cell harvesting procedure follows: the cells were treated with 0.075 M KCl prewarmed to 37°C for 18 min and fixed in acetic acid and

methanol (1:3). The slides were prepared by air-drying method. The slides were stained with 5% Giemsa for mutagen sensitivity assay whereas for CAs, sister chromatid exchanges (SCEs) and cell cycle kinetics study differential staining procedure was followed.

Differential staining

This was carried out following the method of Goto *et al.*[25] Slides were treated for 10 min with Hoechst 33258 (50 μg/mL) at room temperature in dark, rinsed in distilled water, mounted in 2XSSC (NaCl-Na-Citrate, pH 6.8) and kept in sunlight for 30–40 min depending on the intensity of sunlight. After rinsing in distilled water, slides were stained in 2% Giemsa for 3–4 min, air-dried and mounted on synthetic medium.

Determination of GSH-level

The level of total GSH in PBL was estimated by the method of Akerboom and Sies.[26] Lymphocytes were separated out from heparinized whole blood on a Ficoll-hypaque density gradient by the method of Boyum.[27] Freshly collected lymphocytes were washed into ice-cold 0.1 M phosphate-buffered saline solution (pH 7.4) and the volume was made up to 1 mL. Cells were counted in a haemocytometer and lysed by alternate freezing and thawing three times at 10°C and room temperature, each for 10 min. The lysed cell suspension was centrifuged in a Beckman model J2-HS centrifuge (6500 g) for 5 min at 4°C. The supernatant was de-proteinized using 100 μL ice-cold 10% 5-sulphosalicylic acid with intermittent shaking. The tubes were kept on ice for 10–15 min and the acid precipitable proteins were removed by centrifuging at 6500 g at 4°C for 15 min. The supernatant was immediately used for GSH estimation. 50 μL sample suspension was added into 1 mL buffer (0.1 M EDTA phosphate buffer, pH 7.0). Then 50 μl NADPH (4 mg/mL), 20 μl DTNB (1.5 mg/mL) and 20 μL GSH reductase (6 units/mL) were added. The contents were mixed and the optical density of the samples was measured continuously for 5 min at 412 nm by UV-visible spectrophotometer (Beckman model DU-640) relative to a blank without cell extract. A standard curve was prepared from a stock solution of 10 mM GSH (3.1 mg/mL) in 5% 5-SSA diluted to 100–1000 nmol.

Western blot analysis

5×10^6 lymphocyte cells were lysed in radioimmuno precipitation buffer and the amount of protein was determined using the bicinchonic acid protein assay. Equal amounts of protein (80 μg) from each sample were loaded in each well, and equal loading was verified by immunoblotting with actin antibodies. Electropheresis was performed in 10% polyacrylamide separating gel and 5% stacking gel. Proteins were transferred to a 0.45 μm nitrocellulose membrane (Millipore) at 30 V constant voltage for 5 hours using Bio-Rad Trans Blot Cell. After overnight incubation at 4°C in blocking solution of 5% nonfat dried milk mixed in TBST buffer pH 7.6 (1 M Tris·Cl, 5 M NaCl and 0.05% Tween 20), membranes were probed with a 1:1000 dilution of a mouse monoclonal antibody against anti-p53 DO-7 (Neo-Markers, Fremont, USA) in TBST with 5% NFDM for 1 hour. A primary antibody for β-actin (anti-actin ACTN05; Neo-Markers, Fremont, CA, USA) 1:5000 dilutions, was also used to control for sample loading.

Blots were washed three times for 10 min each in TBST and incubated with secondary antibody (alkaline–phosphatase conjugated anti-mouse IgG, 1:2000; Bangalore Genei, Bangalore, India) for 1 hour at room temperature. After extensive washing the blot was immersed in 4 mL substrate solution of BCIP/NBT (5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium – Bangalore Genei, Bangalore, India). Within 20 min bands developed sufficient colour.

Scoring and statistical analysis

Slides were coded in random and metaphases were categorized as in first, second and subsequent division cycles based on their differential staining patterns. Chromosome aberrations were scored from first cycle metaphases (M1) only. Metaphase cells with differentially stained chromatids from each human sample were studied for evidence of SCE. The cell cycle data were presented as average generation time (AGT) which is the ratio of BrdU duration (hours) and replicative index (RI), where $RI = (1 \times M1 + 2 \times M2 + 3 \times M3)/\text{number of cells}$. For mutagen sensitivity assay CAs were examined from coded slides.

The statistical significance of the difference between the chewers and the NC for the frequency of SCEs and AGT was evaluated using Student's *t*-test and for first cycle metaphases (M1) 2×2 contingency χ^2-test was used. To compare the intracellular distribution of SCEs within individual group of NC and chewers, the dispersion coefficient H[28] which is the ratio of the sample variance to the sample mean[29] was analysed.

The statistical significance of the difference between NC and HC for the frequency of aberrant metapahses induced by BLM was evaluated by 2×2 contingency χ^2-test and for different types of aberra-

tions a simple χ^2-test was used. The difference of GSH level between chewers and NC was evaluated using Students' *t*-test.

Results

Cytogenetical parameters

The data of the frequency of CAs, SCEs and first, second and subsequent cycle metaphases in NC, MC and HC are presented in Table 1. CAs are mainly of chromatid break type and for this reason they are not shown here. Although the frequency of aberrant metaphases in HC is not significantly increased with respect to NC, it clearly shows a positive tendency of induction of aberrations in HC. The frequency of SCEs in HC is significantly more than NC whereas in MC there was a tendency of enhancement since five out of ten samples showed more than 6 SCEs per cell which is not so with NC. Regarding the distribution of SCEs per cell, HC showed more cells having 11 or more SCEs with respect to NC and MC. The dispersion analysis indicated that the distribution of SCEs in all the categories did not deviate from Poisson distribution. The frequency of M1 was significantly

Table 1 Frequency of chromosome aberrations, sister chromatid exchanges and cell cycle kinetics in non (N), moderate (M) and heavy (H) chewers

Donor # /Age	*TM_1*	*Abt.M (%)*	*Cells scored*	*SCE/cell*	*SCE/M (%)*			*H*	*TM_2*	*M1 (%)*	*AGT (H)*
					0–3	*4–10*	*≥ 11*				
Nonchewer											
1/24	054	02	29	5.75	21	69	10	1.68$^{\$}$	132	54	45.0
2/33	057	02	34	6.12	12	85	03	0.82	129	52	44.2
3/33	090	02	32	5.16	16	82	02	0.73	251	43	37.9
4/50	112	02	32	5.54	16	84	00	1.06	257	47	38.7
5/37	100	01	39	5.94	13	87	00	0.66	196	52	44.8
6/27*	085	02	55	5.88	13	84	03	0.80	242	46	44.4
7/33*	095	01	34	5.90	03	97	00	0.85	278	34	37.5
8/30*	059	02	33	5.21	25	72	03	1.17	142	42	42.9
9/25*	074	01	37	4.62	32	65	03	1.16	230	32	37.3
10/28*	085	02	32	5.47	19	78	03	0.88	145	60	48.1
Mean ± SEM		02 ± 0.2		5.56 ± 0.1	17	80	03	0.98		46 ± 2.8	42.1 ± 1.2
Moderate chewer											
1/25	068	03	47	6.40	13	83	04	0.92	234	41	40.5
2/27	094	02	38	4.42	39	61	00	1.33	219	53	44.8
3/31	096	01	36	5.76	14	86	00	0.85	244	45	41.9
4/26	118	01	42	5.48	21	79	00	0.71	236	52	45.6
5/32	093	03	35	6.86	03	89	08	1.14	156	61	49.3
6/29	092	03	27	6.48	07	93	00	0.59	159	61	46.2
7/41	088	03	31	5.10	16	80	04	0.93	146	63	48.9
8/28	105	03	32	5.97	16	82	02	0.88	225	46	41.9
9/36	097	02	35	6.26	17	83	00	0.93	193	51	42.9
10/30*	109	02	36	6.83	06	94	00	0.62	234	47	42.4
Mean ± SEM		02 ± 0.3		5.96 ± 0.3	15	83	02	0.89		52 ± 2.4^{c}	44.4 ± 1.0
Heavy chewer											
1/35	112	03	52	5.42	25	69	06	1.19	242	49	41.6
2/46	112	04	24	8.38	00	79	21	1.09	163	70	52.6
3/39	093	04	29	8.72	07	69	24	1.45	171	58	46.8
4/32	091	02	32	7.03	09	87	04	0.84	156	58	48.0
5/50	110	03	28	6.71	11	71	18	1.17	159	71	53.3
6/33	088	03	26	6.88	08	80	12	1.10	138	67	51.1
7/34	075	04	23	7.61	09	69	22	1.74$^{\$}$	134	57	47.4
8/29	079	03	32	6.34	09	85	06	1.18	149	55	45.3
9/70	086	05	24	7.58	00	80	20	1.40	129	73	53.3
10/27	093	03	25	6.60	08	88	04	0.72	154	64	48.0
11/37	067	03	44	11.02	05	41	54	1.65$^{\$}$	160	45	43.4
12/49	076	04	35	6.94	02	89	09	0.84	146	52	43.9
13/42	110	04	28	7.89	09	65	26	1.26	159	72	53.7
14/28*	123	03	42	5.35	19	81	00	0.73	232	55	45.3
15/60*	105	04	26	5.65	23	73	04	1.15	192	60	47.7
Mean ± SEM		04 ± 0.2		7.12 ± 0.4^{a}	10	75	15	1.18		60 ± 2.3^{c}	48.1 ± 1.0^{b}

* Females; TM_1/TM_2, Total metaphases for aberration/cell cycle kinetics; Abt.M, Aberrant Metaphases; M1, First cycle metaphases; AGT, Average generation time; H, Dispersion coefficient; $, Significantly different at $\alpha = 0.05$ from poisson distribution.
[a] $P < 0.05$, [b] $P < 0.001$, Students' *t*-test compared to nonchewer controls.
[c] $P < 0.01$, 2×2 χ^2 contingency test compared to nonchewer controls.

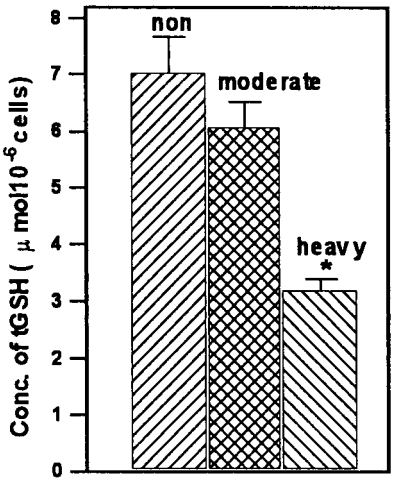

Figure 1 Levels of total GSH in kwai chewers' and nonchewers' peripheral blood lymphocytes Values are mean ± SEM of group of at least 10 donors per dose-point. *$P < 0.05$ as compared with nonchewers control.

increased in HC with respect to NC, however, in MC it showed a positive tendency to increase. Similarly the value of AGT increased appreciably in HC.

Determination of GSH level

The mean level of total GSH in blood lymphocytes of donors is shown in Figure 1. The GSH concentration in NC was ranged from 5.06 to 10.33 μmol 10^{-6} cells with an average of 7.05 ± 0.62 μmol 10^{-6} cells. The GSH concentration in HC was significantly depleted to 55% of the NC while in MC the depletion was marginal.

Mutagen sensitivity

Mutagen sensitivity values were obtained from 7 NC and 5 HC. Table 2 shows that the range of the frequency of aberrant metaphases was 17 to 24% with a mean 20% after exposing the G2 lymphocytes to BLM in NC whereas in HC the range was 20 to 36% with a mean 28%. Both the frequency of BLM induced chromatid break and deletions were increased in HC, however, the extent of increase with respect to deletions was significant. Regarding the distribution of aberrations per cell it is clear from Table 3 that the number of cells having four or more than four were much higher in HC than NC.

Western blot analysis

Representative results of Western blot analysis are illustrated in Figure 2. The p53 protein was significantly more expressed in HC than NC. Lymphocytes of NC were p53 negative ($n = 4$) or weakly positive ($n = 4$) in Western blot analysis under our experimental condition.

Discussion

The present data show higher DNA damage, delay in cell kinetics, p53 expression and lower GSH-level in HC than NC. The inclusion of chewing tobacco, spices or perfumes is avoided during chewing of kwai with lime and betel-leaf. Thus this unique situation favors an investigation into the length of exposure of the oral and esophageal mucosa to chemicals which are released from the BN and leaf during the course of a day. Considerable interindi-

Table 2 Induction of chromosome aberrations in non (N) and heavy (H) chewers with bleomycin

Donor # /Age	*Abt.M (%)/TM*		*Chd.bk. (%)*		*Del (%)*	
	Untreated	*BLM*	*Untreated*	*BLM*	*Untreated*	*BLM*
Nonchewer						
1/24	02/120	17/105	02	10	00	10
2/33	01/107	19/126	01	13	00	21
3/33	02/132	18/118	02	19	00	19
4/50	02/145	24/113	02	37	00	27
5/37	01/143	23/075	01	25	00	25
6/27*	01/141	17/099	01	21	00	19
7/33*	02/197	20/127	02	22	00	24
Mean ± SEM	02 ± 0.2	20 ± 1.1	02 ± 0.2	21 ± 3.3	00	21 ± 2.1
Heavy chewer						
2/46	04/100	29/134	04	20	01	39
3/39	05/079	24/125	05	17	00	41
5/50	04/120	20/123	04	19	00	24
12/49	04/134	31/108	04	28	00	36
13/42	05/120	36/085	05	54	00	46
Mean ± SEM	04 ± 0.2	28 ± 2.8[a]	04 ± 0.2	28 ± 6.9	0.2 ± 0.2	37 ± 3.7[b]

* Females; Abt.M/TM, Aberrant metaphases/Total metaphases for aberration.
[a] $P < 0.001$,2x2 contingency χ^2 test compared to nonchewer controls.
[b] $P < 0.001$, χ^2 test compared to nonchewer controls.

Table 3 Frequency distribution of aberration in BLM treated lymphocytes of non (N) and heavy (H) chewers

Donor # /Age	*Abt.M (%)*	*Aberration/cell (%)*				
		1	*2*	*3*	*4*	*> 4*
Nonchewer						
1/24	17	61	22	11	06	00
2/33	19	42	38	20	00	00
3/33	18	48	14	23	05	10
4/50	24	26	33	22	04	15
5/37	23	12	53	29	06	00
6/27*	17	35	41	18	06	00
7/33*	20	52	44	04	00	00
Mean		39	35	18	04	04
Heavy chewer						
2/46	29	23	21	20	18	18
3/39	24	37	29	20	07	07
5/50	20	48	24	04	16	08
12/49	31	32	29	18	12	09
13/42	36	23	23	16	19	19
Mean		33	25	16	14	12

* Females

vidual variations have been observed regarding the dose and duration of chewing kwai and occurrence of the disease. In the present study, the HC were older (mean age 41 years) than the other groups (mean age for NC and MC are 32 and 30 years, respectively) and had a longer duration of chewing habit. However, reports regarding the effect of age and sex on the frequencies of SCE and CA were contradictory[29,30] and also have no significant influence on the frequency of cytogenetic endpoints in question.[31] Here, due to the small sample size in each group, the evaluation of the effect of these confounding factors will not be meaningful.

In this study, it was attempted to explore the possible utility of a combination of cytogenetic endpoints in relation to endogenous GSH level and p53 expression in assessing the extent of genomic damage caused by the habit of kwai chewing on non-target tissue like PBL in chewers not suffering from cancers. It has been reported that the frequency of lymphocytic SCE was elevated in BN chewers and oral cancer patients in comparison with NC controls.[32] It has also been proposed that the CA frequency in PBL may serve not only as a biomarker of mutagen exposure, but also as a biomarker of genetic damage of relevance for carcinogenic processes.[33] Therefore, in this study we analysed the genotoxic effect in the PBLs, which can be obtained easily, and all these cytogenetical parameters are considered to be sensitive indicators of DNA damage which increases the risk of cancer and genetic ill health.[34,35]

Alkaloids and ployphenols of the kwai have been the main suspect for delivering carcinogenic chemicals to the masticators[1,36] however, the ingredients of betel-leaf also show the mutagenic, carcinogenic and gene convertable potentialities.[37] Due to the presence of lime in kwai preparation, BN-chewers' saliva typically changes from neutral to an alkaline condition which could be ideal for releasing ROS species.[38] Nair *et al.*[39] noted that H_2O_2 and superoxide radicals are produced during autoxidation of BN-ployphenols when the pH level is greater than 9.5. Another important component of BN and betel leaf is the transition metal ions such as Cu^{2+}, Mn^{++}, Fe^{2+} and Fe^{3+}, promoting the production of ROS by BN.[39] Since ROS are important in the initiation and promotion of cancer[40] therefore, it seems that all the components generating from the kwai mixture have the potentialities of cancer induction.

It has been suggested that the metabolic activation may produce a variety of BN-specific nitrosamines which could be the primary cause of oral mucosal lesions.[41] Biochemical studies have found that ARC and arecaidine react with thiol groups both *in vivo* and *in vitro* to produce cysteine 3-alkylation adducts.[42] This may explain the cause of the depletion of GSH in oral fibroblasts and keratinocytes[43] and buccal mucosa of betel-quid chewers[44] and may render the cells susceptible to potential further attack by other BN components or environmental toxicants.

The cell kinetics of PBLs of HC in culture conditions showed significantly slower progression than NC. It has been reported that BN-extract can decrease the clonal cell growth of buccal epithelial cells[45] and fibroblast cells.[46] Earlier ARC showed the ability of induction of delay in cell kinetics in mouse bone marrow cell[6] and similar observation was made by others in human gingival fibroblasts.[43] It is known that ARC, by losing only one of its

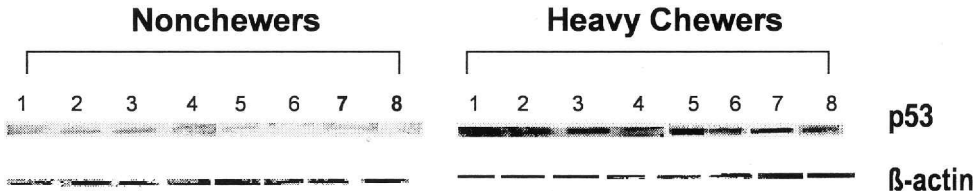

Figure 2 The level of p53 protein in kwai chewers' and non-chewers' peripheral blood lymphocytes. Two sets of experiment have been performed. P53 protein was detected in cell lysates by immunoblotting using monoclonal antibody PAb8.

methyl group[47] may bind with nucleic acid and protein[42] and by doing so it could induce delay in cell kinetics. In addition lower endogenous GSH in PBLs of HC could also be responsible for the delay since cellular GSH are important for cell proliferation.[41]

The elevation of SCEs in PBLs observed in the present study are in accordance with the reports of others.[31,48] The dispersion analysis indicated that the distribution of SCE per cell in both MC and HC showed Poisson distribution which is an indication that the SCE-induction in those samples was due to DNA damage since the DNA damaging agent that induces SCE fits well with the Poisson distribution.[49] The induction of CA was also marginally increased in HC than NC which could also be explained on the basis of the clastogenic potential of kwai components.

A novel aspect of the present study is the analysis of the influence of BLM treatment on chromatid break induction in G_2-phase of PBLs in NC and HC to determine individual susceptibility to genotoxic/carcinogenic assaults. The basis of this mutagen sensitivity may reflect either an underlying DNA repair deficiency or factors which control susceptibility to initial clastogenic influences.[24] BLM induces chromosomal damages through the generation of free radical oxygen and thus, is reflective of one measure of BN-induced damage. HC showed higher induction of chromatid breaks than NC. The number of cells having four or more than four breaks were increased in HC. It was shown previously that there is an increase in the mean level of BLM-induced chromatid breaks per cell in cancer patients compared to healthy control.[50,51] Risk assessment in this study suggest an interaction between carcinogen exposure and mutagen sensitivity measures, risk estimates being higher in those individuals who both consume kwai and express sensitivity to free radical oxygen damage *in vitro*.

It has been reported that a tumour suppressor gene like p53 is effective in protecting cells against DNA damage induced by various agents including ionizing radiation,[52,53] by blocking the damaged cells at the G1 checkpoint and then presumably allowing time for DNA repair.[54] Thus the present observed delay in cell proliferation in HC could partly be explained as higher induction of DNA damage and therefore, more time was required for repair of such damage. If this is true then HC should show higher level of p53 expression and the present result indeed showed higher p53 level in HC than NC. Elevated level of wild type p53 protein after DNA damage induced by radiation or actinomycin D in ML-1 myeloblastic leukemia cells has been reported.[55] Increased expression of p53 protein has also been reported in head and neck squamous cell carcinoma[56] and also in oral dysplastic lesions, suggesting that p53 alteration is an early event in oral oncogenesis.[21] With the concept that carcinogenesis is a multistep process, our findings are consistent with the hypothesis that alteration of p53 protein may play an important role in the early phases of oral carcinogenesis.[57] However, in the present study the elevated level of p53 protein was analysed in the non-target tissue of kwai chewers and therefore, p53 over accumulation could become a potential intermediate biomarker for risk assessment as it was proposed by earlier workers.[57,58]

The possible use of biomarkers representing intermediate steps in the pathway from exposure to disease to estimate the risk of cancer in human populations has gained increasing attention.[59] Hitherto, only cytogenetic biomarkers have been validated.[60] From this study it seems that besides cytogenetical parameters, the level of endogenous GSH and the level of p53 protein could act as effective biomarkers for kwai chewers.

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