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Papers

Micronuclei and Carcinogen DNA Adducts as Intermediate End Points in Nutrient Intervention Trial of Precancerous Lesions in the Oral Cavity

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In cancer chemoprevention trials, biomarkers as intermediate end points have gained importance. A variety of biomarkers have been proposed as intermediate end points for upper aerodigestive tract cancers. This study was aimed at studying the frequency of micronucleated cells and carcinogen DNA adducts as indicators of DNA damage and intervention end points in chemoprevention trials.

Reverse smokers of chutta (rolled tobacco) from four villages numbering 298 in total were selected. Out of these, 150 were supplemented with four nutrients (vitamin A, riboflavin, zinc and selenium) and 148 controls received placebo, one capsule twice a week for 1 year. Slides of buccal smears were prepared and stained with Fuelgen reaction and counterstained with Fast Green and examined microscopically for the presence of micronucleated cells. Oral cell washings were collected and centrifuged. The DNA adducts were evaluated by the ^{32}P post-labelling assay method. Protein and RNA free DNA (adducted) isolated from the cells was digested with MN/SPD and the DNA adducts isolated by the butanol enrichment procedure. The DNA adducts were identified and quantitated by multidimensional chromatography on PEI-TLC sheets by screen enhanced autoradiography and presented as RAL (relative adduct labelling) values.

Both the micronuclei and DNA adducts were significantly elevated in subjects with lesions. At the end of 1 year the frequency of micronuclei decreased significantly ($P<0.001$) in the supplemented subjects with or without lesions. The DNA adducts in the supplement group at the end of 1 year also reduced significantly. The adducts decreased by 95% in subjects with all categories of lesions and by 72% in subjects without lesions. No such effects were noted in the placebo group.

The two biomarkers investigated in the case study appear to be modifiable by the administration of micronutrient supplements. The results are in agreement with the clinical response and suggest that suppression of genetic damage was consistent with clinical remission.

In the study, a cocktail of micronutrients was administered and as such no comments can be made as to the relative benefit of each of the nutrients. However, these biomarkers used in addition to the clinical response of the precancerous lesions can be valuable measures to arrive at beneficial impacts.

Keywords: biomarkers, micronuclei, DNA adducts, ^{32}P post-labelling, oral cancer

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INTRODUCTION

IT IS important and necessary to prevent damage due to environmental exposures either by eliminating the aetiological factors or decreasing their biological effects on the body. As DNA interactions with chemicals are recognised as the primary step in cancer initiation, more emphasis is being given to the methods which detect genotoxic activity, particularly in humans [1]. Biomarkers as intermediate end points in chemoprevention trials are attracting the attention of scientists, particularly for field cancerisation studies [2]. A variety of different biomarkers have been proposed as intermediate

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end points for upper aerodigestive tract cancers [3]. Stich and coworkers have used micronucleated exfoliated cells and carcinogen DNA adducts as indicators of DNA damage and intervention end points in chemoprevention trials [1, 4].

There is enough evidence in the literature to support the hypothesis that micronutrients have a significant role to play in the prevention of precancers and cancers [5, 6]. As oral cancers predominate in India [7] and several precancerous lesions are encountered, both due to chewing and smoking tobacco [8], we envisaged a chemoprevention trial in a high-risk group of reverse smokers. This trial was initiated to assess the efficacy of a cocktail of micronutrients, namely, vitamin A, riboflavin, zinc and selenium on precancerous lesions over the palate which occur as a result of the peculiar habit of reverse smoking of "chutta" (rolled tobacco leaf) in Andhra Pradesh, India [9].

This study reports the effect of micronutrients on genotoxic damage such as micronuclei and DNA adducts in oral epithelial cells obtained from subjects with and without precancerous lesions.

SUBJECTS AND METHODS

Clinical

A complete description of the study design and conduct has been reported previously [10]. A group of high-risk reverse smokers prone to palatal cancers with several precancerous lesions over the palate participated in the study. A total of 298 subjects, 79 males and 219 females aged between 25 and 70 years (66% with lesions and 34% without) were distributed equally in placebo and nutrient-supplemented groups. Palatal lesions were mapped on nine arbitrary zones and clinical photographs taken to facilitate assessment of clinical response. The white (40%), red (50%) and combination patches (10%) greater than 5 mm in diameter were chosen for intervention.

All the subjects underwent complete oral examination initially and after 12 months of the clinical trial. A simple questionnaire was administered to record their habits of smoking and chewing. Dietary intake was quantitated in a subsample and random blood samples collected and analysed for nutrients in the blood, the results of which have been reported previously [10]. The subjects on supplements received vitamin A, zinc, riboflavin and selenium for 1 year. The doses of the micronutrients were changed every 3 months to avoid toxicity, as shown in Table 1. Those on placebo received identical capsules containing lactose.

The subjects who participated in the study were smoking unprocessed tobacco rolled into beedis, locally known as chutta, of 10–12 cm in length with the lit end inside the mouth. Such habits in a majority were initiated at a very young age (< 15 years).

Table 1. Nutrients supplemented and the dosage schedule

	Months		
Nutrients*	1–4	5–8	9–12
Vitamin A (IU)	25 000	10 000	25 000
Riboflavin (mg)	50	15	30
Zinc (mg)	12.5	25	25
Selenium (μg)	100	50	50

*Administered bi-weekly

Cytological

From 40% of the subjects participating in the trial, exfoliated cells were obtained by swabbing the oral mucosa with a moist, wooden spatula. Prior to the sampling, individuals were asked to rinse their mouth with water to remove tobacco fragments and/or food particles. Cells were transferred directly to a precleaned microscope slide, air-dried and fixed by spraying the slides with Mercofix (Merck, Germany) and transported to the laboratory. The smears were stained with Feulgen reaction and counterstained with Fast Green. One thousand cells were screened in each preparation for counting the micronuclei. The procedure and criteria for micronuclei are as described by Stich and Rosin [11].

Biochemical

Exfoliated cells of the entire buccal cavity were also collected on two consecutive days from 50% of the subjects by asking them to rinse their mouth with saline. Mouth washings were collected in test tubes and centrifuged. The cells collected were transported at −20°C to the laboratory and processed for DNA adducts.

DNA adducts were quantitated by the ^{32}P post-label assay as developed by Randerath and associates [12] and modified by Gupta [13]. The DNA was isolated from the epithelial cells by solvent extraction, after enzymatic digestion of protein and RNA [14]. DNA concentrations were estimated spectrophotometrically using a value of 20 A 260 units of DNA/mg.

A known aliquot of DNA was hydrolysed using micrococcal nuclease and spleen phosphodiesterase to deoxyribonucleoside 3-monophosphates [15]. An aliquot of this total DNA digest was diluted and used for the estimation of total nucleotides by one-dimensional TLC in the ^{32}P post-labelling procedure. The remaining digest was used for the estimation of adducted nucleotides by the enrichment procedure of Gupta [13] which involves enrichment of adducted nucleotides with 1-butanol in the presence of the phase transfer agent tetrabutyl ammonium ions. The transfer of ^{32}P post-label to the 5-hydroxyl end of the adducted nucleotide to form 3-5-biphosphates was completed as described below, followed by separation and detection of adducts by high resolution multidimensional chromatography followed by screen enhanced autoradiography. The quantitation of adducts was performed by Cerenkov counting. Locally available X-ray sheets were coated with MN-300 cellulose. The sheets were subjected to dilute acid, alkali and petroleum ether treatment and finally washed with distilled water and dried and coated with a 5% polyethyleneimine–MN-300 cellulose slurry. After overnight drying, the sheets were exposed to deionised water followed by air drying.

ATP was synthesised by substrate level phosphorylation of ADP using carrier-free ^{32}P-orthophosphoric acid by the procedure of Johnson and Walseth [16]. The ATP synthesised was tested in one-dimensional TLC and identified by autoradiography. The specific activity of ATP obtained in the study was approximately 3000 Ci/mmol based on polynucleotide kinase catalysed phosphorylation of a known amount of DNA nucleotides. For labelling purposes 100–200 μCi of gamma ^{32}P ATP was used for 10 μg of DNA digestion. Relative adduct labelling was calculated as described by Gupta [13]. As the DNA yield from the cells of single samples was not adequate, pooling of these samples according to the lesion status was carried out prior to MN/SPD enzymatic digestion and adducts estimated in the pooled samples.

Statistical

The difference in the mean values between groups were compared by Student's *t* test.

RESULTS

Lesions

The clinical examination performed by two medical doctors indicated complete regression in 57% of subjects on supplements and 8% on placebo. New lesions appeared in only 12% of subjects on supplements but 48% on placebo. The clinical criteria for response and other particulars are described in a separate communication [10].

Micronuclei

The results of micronuclei determination are indicated in Table 2 and Fig. 1. The frequency of micronuclei among those with lesions was significantly higher as compared to those without lesions of the oral cavity (Fig. 1). On supplementing nutrients for a period of 1 year, there was a significant decrease in the frequency of micronuclei in subjects with and without lesions. But in those receiving placebo, there was a significant reduction in micronuclei only in the lesion group. However, the percentage fall was much higher in the supplemented group (60%) as compared to the placebo group (35%). The frequencies between the placebo and supplement groups were also significantly different, being lower in the latter group ($P<0.001$). In Table 2, micronucleated cells in red, white and combined lesions show that all subjects with lesions respond with a reduction in the frequency, the reduction again being much higher in the supplement group.

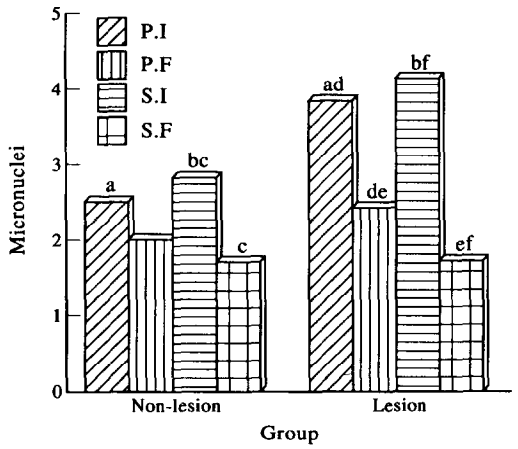

Fig. 1. Micronuclei scored per 1000 intact cells of the oral cavity. PI=placebo initial; PF=placebo final; SI=supplement initial; SF=supplement final. Same superscripts are significant, a–f: $P<0.001$.

DNA adducts

The DNA adducts measured in pooled estimates are given in Table 3. There were significantly more adducts in the lesions compared to the non-lesion group. Infact when the lesions were categorised and the DNA adducts in each of them separately analysed, it was observed that the red lesions had a level of 46.7 ± 24.29 fmol/μg DNA (25 individuals or six pooled estimates) as compared to 14.1 ± 5.4 fmol/μg DNA in the non-lesion group (43 subjects or six pooled estimates, $P<0.001$). After therapy, appropriate sets of data were compared. It is obvious that in subjects who received supplements, irrespective of whether or not the lesions were present, a significant drop in DNA adducts was observed, as compared to the initial values; while on placebo, no significant changes were observed either in the lesions or in the non-lesion group. The comparisons of post-treatment DNA adducts between placebo and supplement groups showed that those who had no lesions in the latter group had the least amount of adducts (Table 3 and Figs 2, 3).

Table 3. Buccal cell DNA adducts in reverse smokers (fmol/μg DNA)

Groups	Initial	Final	
		Placebo	Supplemented
Non-lesion	14.1 ± 5.37^{a} [43] (6)	$12.31\pm5.73^{c,e}$ [10] (2)	$3.9\pm0.85^{a,c}$ [24] (6)
Lesion	33.4 ± 21.85^{b} [51] (16)	22.5 ± 10.83^{d} [42] (12)	$1.7\pm0.65^{b,d,e}$ [8] (4)

Values are mean ± S.D.
Same superscripts are significant, a: $P<0.001$; b, d, e: $P<0.02$; c: $P<0.01$.
[ ] = no. of subjects; ( ) = no. of pooled estimates.

DISCUSSION

Cancers at certain sites are associated with several life style factors. Of these, dietary factors may act as initiators (causal), enhancers (promoters) and protectors (inhibitors) in the diet

Table 2. Micronuclei in buccal epithelial cells (micronuclei/1000 intact cells)

Type of lesion	Placebo		Supplement	
	Initial	After 1 year	Initial	After 1 year
White	3.55 ± 0.50^{a} (11)	$2.18\pm0.72^{a,c}$ (11)	3.58 ± 0.82^{b} (19)	$1.67\pm0.58^{b,c}$ (18)
Red	3.67 ± 1.07^{d} (9)	$2.63\pm0.86^{d,c}$ (8)	4.13 ± 0.61^{e} (23)	$1.55\pm0.59^{e,f}$ (21)
Combined	4.57 ± 0.49^{g} (7)	$2.43\pm1.05^{g,i}$ (7)	4.6 ± 0.49^{h} (10)	$1.6\pm0.49^{h,i}$ (10)

Values are mean ± S.D.
No. of subjects indicated in parentheses.
Same superscripts are significant, a, b, e, f, g, h: $P<0.001$; c, d, i: $P<0.05$.

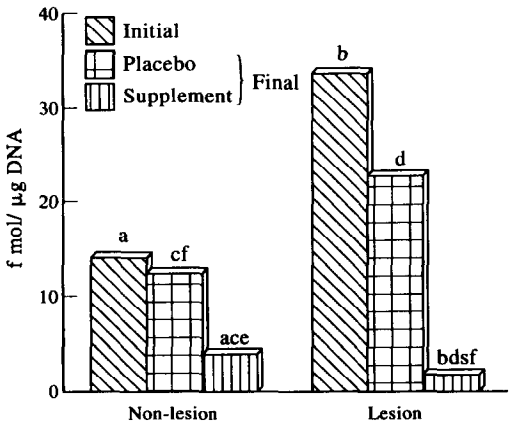

Fig. 2. Buccal cell DNA adducts in reverse chutta smokers. Comparison between initial and final (12 months) value as well as between placebo and supplemented lesions and non-lesions. Same superscripts are significant, a: $P<0.001$; c, e: $P<0.01$; b, d, f: $P<0.02$.

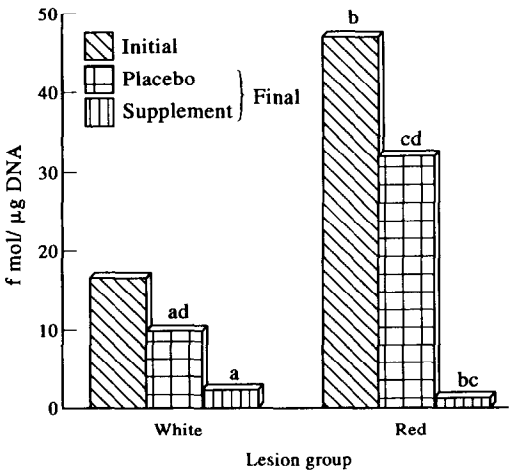

Fig. 3. Buccal cell DNA adducts in reverse chutta smokers. Comparison between initial and final (12 months) value as well as between lesions in placebo and supplement groups (white patches and red areas). Same superscripts are significant, a, c, d: $P<0.001$; b: $P<0.05$.

[6]. As cancer cure, unless detected and treated earlier, is not a feasible proposition, nutrition-based intervention strategies targeted at specific sites in high-risk groups appears to be a better solution [17]. Results of our clinical observations reported elsewhere coupled with more objective quantifiable biomarkers presented above, support the concept that dietary factors, more importantly antioxidant nutrients are effective chemopreventive agents, particularly in cancers of epithelial origin.

Intervention trials which use cancer as an end point undoubtedly provide the most accurate, direct and unambiguous interpretation of end results, i.e. prevention of cancer. However, the large sample size required and long periods of observation have awakened a special interest in biomarkers of genotoxicity. These can be used both as markers of exposure and as intermediate end points as surrogates for cancer in chemoprevention trials [1]. The biological monitoring activities which give an indication of adverse effects which precede or are associated with preneoplastic and neoplastic changes, such as micronuclei and carcinogen DNA adducts, appear to be good tools for judging the response to specific agents.

Micronuclei as biomarkers have been widely used as an indicator of elevated risk in mammalian cells, cultured cells or in exfoliated cells and in biopsy samples [1]. Micronuclei are a reflection of clastogenic events and indicate the ongoing process of DNA damage. It has been correlated with cancer risk at several sites, namely oral, oesophagus, cervix, lung and bladder and appears to be an economical procedure. The frequency of micronuclei in chewers of betel quid has been demonstrated to be very high [1]. In the current study, the frequency of micronucleated cells was uniformly elevated in all lesions suggesting a strong biological effect on chromosomes and chromatid fragments, which responded dramatically to micronutrients. The significant improvement in the frequency of micronucleated cells in the placebo group cannot be explained by changes in smoking habits. A questionnaire relating to their chutta smoking habits indicated that they indulge in smoking not less than 5 times/day regularly. It was found to be consistent until the end of our study. Only three subjects, two on supplements and one on placebo, reported reduction in smoking habits, of which two had lesions one in each group. No further change in life style has been reported during this period. It is known that on cessation of radiotherapy, the frequency of micronucleated cells returns quickly to normality [18]. It is possible that factors such as the maturity and the ripening of tobacco leaves and the quantity of tobacco-specific nitrosamines might have varied, giving rise to such conflicting results. Further, as compared to the earlier reports, the frequency of the micronucleated cells appears to be much less in the present study. This could be due to a dilution effect, since the entire buccal cavity was scraped instead of the lesion and the surrounding area. In spite of such variations, since the same subjects served as their own controls, the decline in the frequency in the supplemented group acquires significance. It points to the fact that the antioxidant nutrients have a successful chemopreventive effect against genetic damage.

Biological markers can often reflect exposure doses and its interaction with critical macromolecules such as DNA [19]. Several investigations in the recent past suggest a causal and quantitative relationship between DNA adducts and cancer such as adduct forming capacity and *in vivo* carcinogenicity [20], *in vitro* cell transformation [21], species sensitivity [22] and activation of oncogenes [23]. Therefore, we used the DNA adducts in epithelial cells as a reflection of damage due to polycyclic aromatic hydrocarbons in the unprocessed tobacco.

Earlier studies of Hoffmann *et al.* [24], indicate that the carcinogen load from smoking such unprocessed tobacco is several folds greater than cigarettes. We observed that not only those with lesions, but among the lesions those with red areas, had the highest damage compared to white or mixed lesions. This infact is in keeping with observations of Mehta *et al.* [25] who suggest a high rate of dysplasia (52%) and malignant transformation (7%) in such lesions. This observation therefore assumes considerable significance in terms of its clinical application. Human studies with ^{32}P post-labelling summarised by Beach and Gupta [26] have highlighted the causal and quantitative relationship between different kinds of exposure and DNA damage. With clinical recovery or remission, the damage to DNA decreased even in the presence of continued smoking habits. Unlike the micronuclei, the DNA adducts in

the placebo group did not alter while even those without lesions in the supplemented group registered a significant decrease in damage after 1 year of supplementation.

The estimates of DNA adducts, however, were much higher as compared to previous reports [26]. The higher values are probably due to diagonal radioactive zones which result when many adducts of wide varying polarity are subjected to multidimensional chromatographs [27]. Further methodological issues such as higher background RNA spots, 'I' spots and test tube spots may have contributed to a higher value. It is also possible that factors such as the primitive cooking system used in ill ventilated low thatched-roof houses result in unknown fuel and food exposures. Nevertheless, before and after treatment experimental procedures adopted were identical and, hence, the results and conclusions drawn are valid.

CONCLUSIONS

In conclusion, the two biomarkers investigated in the case study appear to be modifiable by the micronutrient supplements. However, in view of the small sample size and pooled estimates of DNA adducts, the data need to be interpreted with caution. The results are similar to those of Stich and coworkers [4] and micronuclei in oesophageal cancer [28] where suppression of genetic damage was consistent with clinical remission.

The clinical response associated with positive (decrease) impact on genetic damage, suggests that such biomarkers used in conjunction with pre-cancerous lesions can be valuable measures to arrive at beneficial impacts.

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