GENOTOXIC EFFECTS OF RAW BETEL NUT EXTRACT IN MAMMALIAN CELLS AND ITS DNA DAMAGING ACTIVITY IN BETEL NUT CHEWERS WITH RESPECT TO CELLULAR GLUTATHIONE STATUS

By

KABITA KUMPAWAT

SUBMITTED

IN

FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN ZOOLOGY
OF
NORTH-EASTERN HILL UNIVERSITY
SHILLONG-793022

NORTH EASTERN HILL UNIVERSITY
SHILLONG-793022
JUNE, 2002

DECLARATION

I, Kabita Kumpawat, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other university/ Institute.

This is being submitted to the North Eastern Hill University for the degree of Doctor of Philosophy in Zoology.

Head

Head Deptt. of Zoology
School of Life Sciences
NEHU., Shillong-22

Supervisor

Kabita Kumpawat

Candidate

Dedicated to

Mummy and Papa

ACKNOWLEDGEMENT

"God helps those who help themselves". With these words in mind I started this long journey and as I inch my way to the end, I want to pause, reflect back, and express my gratitude to all those whose love and support was enough to keep me going.

My mentor and guide, Dr. A. Chatterjee, who opened the door to genetics. His guidance is a stepping stone to success. His encouragement, constructive criticism gave way to building a strong determination in me and taught me to take life as it comes. Whatever I am today and whatever I will be is because of you sir. Hard-work, devotion and sincerity- no one could have taught me the importance of these virtues better than you. I owe a lot to you sir.

Thanks to the Head, Department of Zoology for providing me all the indispensable laboratory facilities for completing my research work. My sincere thanks to all the faculty of the department of zoology for rendering help and being there whenever I needed them.

I express my gratitude to Dr. Churungoo, Department of Botany, for allowing me to use some instruments. This work would not have been possible without Dr. Vinod Singh, Department of Biophysics for allowing me to use the lyophilizer. I express my thanks to him.

I am also indebted to all the betel-nut chewers who not only generously gave blood and co-operated fully but also showered their best wishes for a good and fruitful outcome. I thank you all.

I deeply appreciate the friendly cooperation by the photographer B.K. Das.

My special thanks to Arpaia, Hillson, Bonnie, Bidhayak and Frederick for unselfishly helping me in searching donors.

I express my deepest sense of gratitude to my lab-mates- Shampa, Christine, Aparajita, Sanjib and Frederick for your unconditional help.

Thanks to Ranjit Singh Chingakham, Department of Biophysics for acquainting me with the lyophilizer.

I specially would like to thank all my friends in Balgito halls of residence for giving me such a homely environment. Friends you all made the place "a home away from home".

My thanks and best wishes to my room-mates Swapnalee and Imnainla who have been there throughout as sisters in my good and bad times. May God bless you.

Thanks to mummy and papa- for all the love, emotional support that you gave. You two are the "wind beneath my wings" and I could soar so high today because of you. You have pushed untapped reserves of strength in me.

I express my deepest sense of gratitude to all my brothers, sisters, sister in-laws, brother-in-laws for their love, affection, patience and realising that my dream is their dream. My thanks to all my nieces and nephews for helping me in their sweet little ways.

I acknowledge the financial support provided by the North Eastern Council (NEC), Shillong and UGC-DRS-III project to the Department of Zoology, North-Eastern Hill University, Shillong.

Throughout my life God kept me in his protective lap and I love and believe in Him even more. I thank you God for everything you gave me in life. I realised that "I can do everything through God who gives me strength".

Thanks.....To all the people who helped me at different times, in different ways.
.....May all your dreams come true,
and this time mine.

Kabita.

CONTENTS

ABBREVIATIONS

5-SSA	5-sulfosalicyclic acid
8-OH-dG	8-hydroxy deoxyguanosine
AAEBN	Acetic acid extract of betel nut
Abt.M	Aberrant metaphases
AEBN	Aqueous extract of betel nut
AGT	Average generation time
ARC	Arecoline
BLM	Bleomycin
BMC	bone marrow cells
BN	betel nut
BNE	Betel nut extract
BQ	betel quid
BSNA	betel nut specific nitrosamines
BSO	L-Buthionine-S-R-Sulfoximine
BUdR	5-bromodeoxyuridine
CAs	Chromosomal aberrations
Chd.bk	chromatid break
Cox-2	cyclo oxygenase
Del	deletion
DMBA	9,10-dimethyl bez (a) antracene
DTNB	5-5'-dithiobis 2'-nitrobenzoic acid
EDTA	Ethyl diamine tetra acetic acid
FH	ficoll hypaque
FPG	flourence plus giemsa staining
G6PD	glucose-6-phosphate dehydrogenase
GGT	γ-glutamyl transpeptidase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	Reduced Glutathione
GSSG	glutathione disulfide
GST	glutathione-S-transferase
HC	heavy chewer
H_2O_2	hydrogen peroxide
HNSCC	head and neck squamous cell carcinoma
HPBLs	Human peripheral blood lymphocytes
i.p.	intra-peritoneal
K_2HPO_4	Dipotassium hydrogen phosphate
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
MC	moderate chewer
M.I.	Mitotic Index
M1	first cycle metaphases

M2	second cycle metaphases
M3	third cycle metaphases
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NBT	nitro blue tetrazolium
NC	Non-chewer
NFDM	non fat dried milk
NG	Nitrosoguanidine
NMPA	N- (methylnitrosamino) propionaldehyde
NMPN	N- (methylnitrosamino) propionitrile
NOC	N-nitroso compounds
$O_2^{\cdot-}$	superoxide radical
OA	oral administration
$OH^{\cdot}$	hydroxyl radical
OSF	oral submucous fibrosis
PHA	phytohaemagglutinin
RBN	Raw betel nut
RBNE	Raw betel nut extract
R.I.	replicative index
RIPA	radio immuno precipitation buffer
ROS	reactive oxygen species
RPMI 1640	Rosewell park memorial Institute 1640
SCEs	Sister chromatid exchanges
SOD	superoxide dismutase
SSC	sodium-chloride-sodium citrate
tGSH	total glutathione
TNB	5-thio-2-nitrobenzoic acid
TSNA	tobacco specific nitrosamines

GENERAL INTRODUCTION

The human body is continuously exposed to chemical carcinogens present in air, food and water supplies. Once in the body, these generally inert chemicals can be metabolically activated to form highly reactive and dangerous compounds. These compounds can bind to DNA, the genetic material of the cell and if not removed by cellular repair enzymes can give rise to mutations and cancer.

Almost 80% of human cancers are believed to arise as a direct consequence of involuntary exposure to mutagens and carcinogens (Muir and Parkin 1985, Tomatis et al 1989). There is however another form of 'purely voluntary' exposure that is responsible for a large percentage of human cancers. Habitual exposure of betel nut (BN) or betel quid (BQ) contributes to a large extent in this category, which has been addressed as a major causative factor of oral cancer. Sanghvi (1981) reported that over 50% of the total cancer incidence in India are caused by betel quid consumption. Epidemiological studies have elucidated that the habit of chewing betel quid (BQ) is associated with an increased risk of oral submucous fibrosis (OSF) and oral cancer (IARC 1985, Kwan 1976). In India oral cancer constituting 9.8% of an estimated 6,44,600 incident cancer cases in males and is the third most common among females in many regions, with age-standardized incidence rates from 7-17/100,000 persons/year (Parkin et al 1997). The incidence rate is higher than the western rate of 3-4/100,000/ year (Macfarlene et al 1994). In North-Eastern parts of India, the BQ typically consists of betel nut (BN), piper betel (betel leaf), and slaked lime without tobacco. Oral cancer has been casually associated with chewing of tobacco with or without betel quid in India and other Asian countries, whereas in Western countries, cigarette smoking and heavy alcohol consumption are the main risk factors. It has been estimated that there are about 600 million BQ chewers living in different regions of the world (Sen et al 1989, Sharan 1996).

Chemical carcinogenesis is now recognized to consist essentially of two steps namely initiation and promotion in most tissues. The first stage initiation results in

the binding of the carcinogen to cellular DNA followed by fixation of the alteration during DNA replication. During the promotion step chronic exposure to certain chemicals not carcinogenic by themselves, result in early appearance of cancer with increased incidence. Since more than 60% of the cancers have important environmental factors in their etiologies, this has generated a lot of interest in chemical carcinogenesis. It is now evident that some chemicals may not be carcinogenic per se but may require metabolic activation. As the number of chemicals recognized as carcinogens increased it becomes evident that these chemicals lacked a common structural feature and that most carcinogens bind to cellular macromolecules like DNA, RNA and proteins. During the last few years metabolic activation of these carcinogens has received attention primarily due to the elucidation of the critical role of biotransformation in chemical carcinogenesis and acute toxic liver injury. Prevention of the binding of these electrophilic or ultimate carcinogens to cellular macromolecules is brought about by glutathione (GSH) a highly nucleophilic compound present in large amounts in the liver. GSH conjugation appears to be the major protective mechanism since GSH depletion precedes morphological signs of liver damage after administration of carcinogenic and hepatotoxic agents believed to exert their action through the formation of reactive metabolites.

COMPOSITION OF BETEL NUT

The chewing of betel quid without tobacco is a habit of great antiquity which is widespread in the orient- Bangladesh, Burma, China, India etc. The first reference to 'betel chewers' cancer was made by Tennet (1860). He mentions in a footnote that Dr. Elliot of Colombo observed several cases of cancers in the cheek pouch and from its peculiar characteristics, he designated the term 'Betel Chewers' cancer. Although the chewing of betel quid is practiced in several different ways in various countries, the major components are relatively consistent and include betel nut, betel leaf (piper betel L) and lime.

Betel nut: It is the fruit of the areca catechu L tree belonging to the family Palmaceae. The areca palm is native to south Asia and is found throughout south and South East Asia and in several Pacific Islands.

Betel- leaf (Piper betel L): Betel vines are cultivated in hot and humid climatic conditions in different parts of India, Indonesia, Malaysia and Ceylon.

Lime: It is manufactured on an industrial scale and is sold as a paste mixed with water in order to release calcium hydroxide (Kandarkar and Sirsat, 1977).

In general the BN is 'cured' or 'refined' before use. The table below shows the major chemical ingredients in the BN before curing. It should be noted that wide variations might occur.

Table1 Constituents of BN endosperm before curing :

Constituents	**Quantity**
Tannins	11.4-26%
Gallotannic acid	18.03%
D- Catechol	3g/800g(0.4%)
Alkaloids	0.15-0.67%
Arecoline	0.07-0.50%
Arecaidine	Small quantity
Guvacine	Small quantity
Isoguvacine	Trace quantity
Arecolidine	Minute quantity
Guavcoline	Minute quantity
Fats	1.3-17%
Sitosterol	Trace quantity
Carbohydrates	
(saccharose reducing Proteins)	47.2-84.5%
Non protein nitrogen	0.22-1.6%
Carotene	5 International Vitamin E Units/100g
Mineral Matter	
Calcium	0.018-0.05%
Phosphorus	0.13-2.53%
Iron	1.5-11.6mg/100g (0.002-0.01%)

(From Raghavan and Baruah, 1958)

The form of BN is highly variable between different parts of the world. The most common type is sun-dried variety, in which unripe or ripe nuts are sundried for several weeks. The other type is raw and wet variety as used in Taiwan and whole of the North Eastern and parts of the southern states of India. The North-East Indian variety of betel-nut, locally called as 'Kwai' is raw, wet and unprocessed (RBN) consumed with betel leaf and slaked lime and it is the raw variety which is rich in alkaloids, polyphenols and tannin compared to the dry one. The table below shows the average composition of the two main varieties of BN: sun-dried and wet. The wet variety is the one that is consumed here by the locals and this type was used for our study.

***Table:* Average constitution of two main varieties of BN.**

Constituents	*Sun-dried (%)*	*Wet (%)*
Alkaloids	0.25	0.25- 0.38
a. Arecaidine	0.10	0.10-0.20
b. Arecoline	0.15	0.18-0.24
c. Others	trace	ND
Ash content	low	very high
Carbohydrates	25	30
Crude fibre	15	18
Fats	12	2.50
Polyphenols	15	ND
Proteins	7.5	18
Tannins	18	ND
Water	25	60

ND=Not determined
From Sharan 1996.

The Khasis of Meghalaya usually chew one quarter of a fresh betel nut and part of a lime covered betel leaf. As a rule, the inclusion of chewing tobacco, spices or perfumes is avoided. Thus, this unique situation favors an investigation into the length of exposure of the oral and esophageal mucosa to chemicals that are released from the BN and leaf during the course of the day (Stich et al 1983).

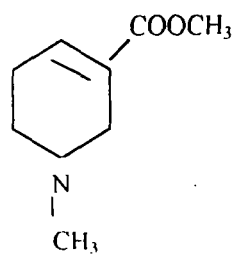
The table below shows the variations in the appearance and disappearance of tannins from the saliva of chewers (Stich et al 1983).

Table: Release of "tannic acids" into the saliva of khasis chewing one quarter of BN and betel leaf with lime (Stich et al 1983).

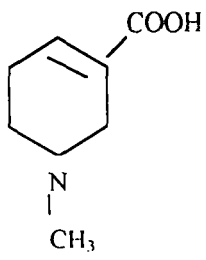
	Tannic acid/ cc saliva			
		Chewing (min.)		
Individual Chewing pattern	Prior to chewing	3	15	25
Vigorous chewing	06	720	19	04
Vigorous chewing	00	1280	21	22
Chewing	11	105	2080	28
Slow chewing	00	65	28	30

BN, the major part of BQ has been the main suspect for delivering carcinogenic chemicals to the masticators (Sharan 1996). Of all the constituents of BN, alkaloids and polyphenols are two groups of chemicals, which could contribute to carcinogenicity (IARC 1985, 1987). The tannic acid fractions from BN also possess appreciable genotoxic activity (Stich et al 1983).

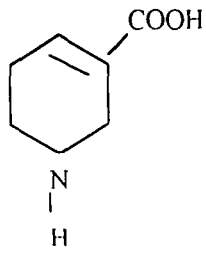
***ALKALOIDS*:** Alkaloids are prime suspects for BN carcinogenesis, a group of reduced pyridine compounds (figure shown below) producing various adducts including cysteine β-alkylation products. Arecoline (1,2,4,5, -tetrahydro-1-methyl-pyridinecarboxylic acid) is the most abundant alkaloid; and other alkaloids present in trace amounts are Arecaidine (1,2,5,6-tetrahyro-1-methyl-3-pyridine carboxylic acid) Guvacine (methyl ester of arecaidine) Guvacoline (methyl ester of guvacine) and Arecolidine.



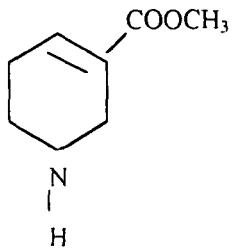
ARECOLINE



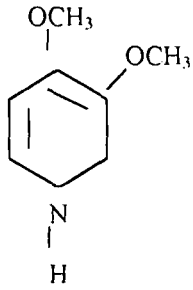
ARECAIDINE



GUVACINE



GUVACOLINE



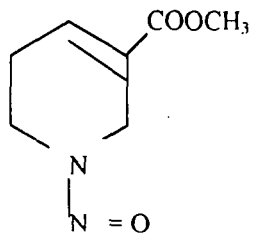
ARECOLIDINE

POLYPHENOLS AND TANNINS: The main polyphenols of BN are catechin, flavanoids, flavan-3: 4-diols, leucocyanidins, and hexahydroxyflavins. These are oxidized in the presence of lime, which imparts the red colour to the saliva. Reactive oxygen species (ROS) like superoxide anion ($O_2^{\cdot}$) and H_2O_2 are produced during autooxidation of BN polyphenols in BN chewer's saliva (Nair et al 1987). ROS can lead to DNA-base damage as 8-OH-dG thymine glycol which are implicated in mutagenesis/ carcinogenesis (Cadet 1994). Gallotannic acid is the predominant tannin of BN.

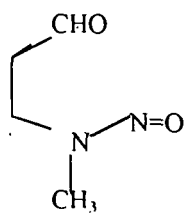
The largest class of potential mutagens present in man's environment are represented by the alkylating agents. Arecoline (ARC) is a monofunctional alkylating agent. It loses one of its methyl groups during metabolism (Boyland and Nery 1969) and binds with nucleic acids and proteins (Nery 1971). The secondary effect of binding is depurination, which may lead to the breakage of the chromosome. Panigrahi and Rao (1982) reported that the secondary effect of ARC potentiates various kinds of chromosomal abnormalities. Alkylating agents

react with macromolecules in the cell such as proteins, RNA and DNA. Extensive protein alkylation can be toxic for a cell due to loss of function of essential proteins. Although DNA alkylation is cytotoxic, after exposure to lower doses the major concern is the mutagenic potency of certain adducts. The reaction with DNA includes the transfer of alkyl groups to the ring nitrogen atoms, the exocyclic oxygen atoms of the bases or the oxygen atoms in phosphodiester bonds (Vogel and Nivard1994).

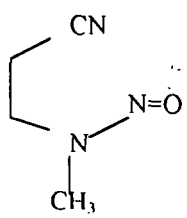
Arecoline, the major alkaloid of BN, under mild nitrosation conditions forms N-nitrosocompounds (NOCs) in vitro (Wenke and Hoffman 1983, Nair et al 1985) and *in vivo*, and is implicated in carcinogenesis (IARC 1985, Castignaro 1988). Saliva samples of BQ/ BN chewers show appreciable concentrations of betel-nut specific nitrosamines (BSNA) and tobacco specific nitrosamines (TSNA) (Wenke and Hoffman 1983, Wenke et al 1984, Nair et al 1985, 1986). Similarly, urine samples of betel nut chewers also show presence of BSNA (Ohshima et al 1989). During BN chewing nitrite and thiocyanate are released in the saliva of chewers and increases salivary pH (IARC 1985). In the presence of nitrite BN alkaloids give rise to N-nitrosamines which interact with DNA, proteins or other targets forming adducts to exert its carcinogenicity activity (IARC 1985, Hoffmann et al 1994). The four areca nut N-nitroso compounds found are N-nitrosoguvacoline, N-nitrosoguvacine, 3-(N-nitrosomethylamino) propionitrile (NMPN) and 3-(N-nitrosomethylamino) propionaldehyde (NMPA) (IARC 1985).



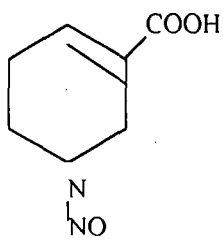
N-nitrosoguvacoline ($C_3H_{10}N_2O_3$)



N-(methylnitrosamino) propionaldehyde(NMPA) ($C_4H_8N_2O_2$)



N-(methylnitrosamino) propionitrile (NMPN) ($C_4H_7N_3O$)

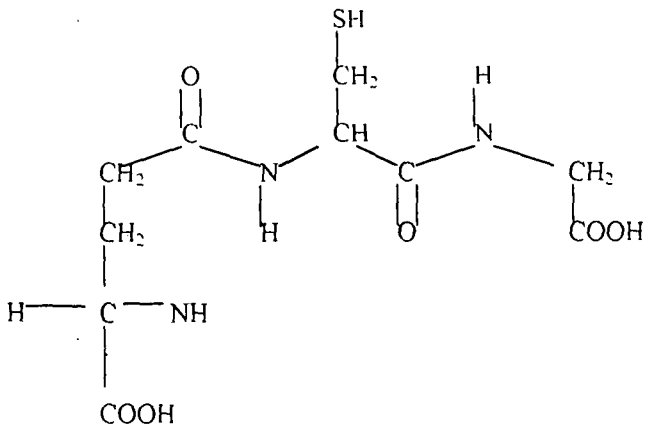


N-nitrosoguvacine ($C_6H_8N_2O_2$)

Arjungi (1976) and Mazumdar et al (1982) have reviewed the toxic and pharmacological effects of BN. Aqueous extract of BN (AEBN) (i.p) increased GSH content and decreased protein –SH in liver, kidney and muscle of Swiss mice (Shivapurkar and Bhide 1978). Both AEBN and ARC increased hepatic DNA and RNA content and stimulated DNA and RNA synthesis in the liver in Swiss mice (Shivapurkar et al 1978, Shivapurkar and Bhide 1979). An increase in the frequency of chromatid breaks and exchanges was observed in culture CHO cells following exposure to aqueous, ethyl acetate or n-butanol extracts of dried BN in the absence of an exogenous metabolic system (Stich et al 1983). BQ chewing increased nitrite levels in saliva, but such increase was not seen when BQ was chewed with tobacco (IARC 1985). The increased frequency of sister chromatid exchanges (SCEs), Chromosomal aberrations (CAs) and micronucleated cells in exfoliated cells of buccal mucosa among BN chewers was reported (Dave et at 1991,1992). The enhancing effects of dietary administration of BN on carcinogenesis in the liver and upper digestive tract were also observed (Tanaka et al 1983). The frequency of lymphocytic SCE was elevated in betel nut chewers and oral cancer patients in comparison to non-chewer controls (Trivedi et al 1995). Such an increase in DNA damage among BN chewers signifies the in vivo genotoxic effects of BN on the non target tissue and its possible role in increasing the risk of cancers at sites other that oral cavity. Nair et al (1992) reported that addition of lime to other BQ ingredients induce the generation of reactive oxygen species (ROS) i.e. superoxide radical ($O^{\bullet}_2^-$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) in the cheek pouch of Syrian Golden Hamsters. According to Sundqvist et al 1989, BN extract decreases cell survival, vital dye accumulation and membrane integrity in a dose dependent manner and it causes both strand breaks and DNA-protein cross-links. Depletion of cellular free low molecular weight thiols also occurs at toxic concentrations.

Continuous exposure of aerobic organisms to pro-oxidant challenges has endowed living cells with efficient and sophisticated antioxidant systems. These can be divided into enzymatic antioxidant and non-enzymatic antioxidant systems. As the most important members of the enzymatic defense systems against oxygen radicals superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPX) have been distinguished (De Zwart et al 1999). Obviously, assaying these enzymes can offer an indication of the antioxidant status of an individual. Besides measuring the enzymatic antioxidant systems in blood samples, non-enzymatic antioxidants can be monitored as well eg, Vitamin E and C, β- carotene, urate, retinyl esters and glutathione (GSH) (Jaeschke 1995). Disturbances in the antioxidant systems might be useful indicators of the susceptibility of subjects to free radical damage. Furthermore, establishing disturbances in antioxidant systems might be useful to decide on starting supplementation with antioxidants in order to prevent oxidative free radical damage and to evaluate the effectiveness of this type of medication. GSH, a naturally occurring tripeptide (L-γ- glutamyl-L- cysteinyl glycine) is a small molecule found in every cell. GSH is present in millimolar concentration (0.5-10mM) and is found throughout the cell, with the bulk in the cytoplasm; subcellular particles, such as the nucleus and mitochondria, having smaller amounts (Meister 1988). GSH is characterized by its reactive group –SH and its γ-glutamyl bond that makes it resistant to normal peptidase activity and gives it its biochemical activity (Moldeus and Jiang 1987). Features of GSH intimately associated with its diverse and important functions include (1) Protection of cells against oxidative damage. (2) Existence of an important component of a system using pyridine nucleotides to provide a reducing atmosphere essential for the integrity of cell membranes. (3) A key role in amino acids transport and multiple metabolic pathways such as the synthesis of proteins, nucleic acids and leukotrienes (Meister and Anderson 1983, Hammerstrom 1981). (4) Regulation of enzyme activation and the immune response (Reed and Fariss 1984) and (5)

Acting as a reservoir of cysteine (Tateishi et al 1977). GSH is also proposed to be involved in the homeostasis and detoxification of metal ions in biological systems (Freedman . et al 1989, Gardner and Fridivich 1993, Kang and Enger 1988). Alterations in the activities of these enzymes may reflect reduced cellular defense and may serve as surrogate markers of many diseases. As GSH is also involved in the regulation and expression of proto-oncogenes and apoptosis (programmed cell death), the development of diseases such as cancer and human immune deficiency may be effected by depleting or elevating cellular GSH levels (Rahman et al 1999).



STRUCTURE OF GSH

The National Academy of Sciences defines a biomarker or biological marker as a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample (ENTOX/TIWET 1996). Silbergeld and Davis, 1994 defines biological markers as physiological signals or inherent or acquired susceptibilities, which provide a new strategy for resolving some toxicological problems. Sensu stricto, biomarker can also be defined as a biological response to a chemical or a group of chemical agents but not the presence of the agent or its metabolite within the body (internal dose) (Walker et al 1996). However, there is no doubt that the measurement of a xenobiotic in a biological system or sample is a bioindicator of

exposure, and thus it could be considered a biomarker. The use of biomarkers is becoming increasingly important in several fields-occupational medicine, epidemiology, genotoxicity, and developmental and reproductive toxicology (Brooks 1999, Albertini 1998, Mattison 1991, Shugart and Theodorakis 1998) and is considered useful in human health risk assessment (Greim et al 1995, Vallyathan et al 1998). Biomarkers are used to measure the exposure of a toxic agent, to detect degree of any toxic response, and to predict the possible effect (Timbrell 1998, Diehl-Jones and Boles 2000). A biomarker should be quantitative, sensitive, specific, and easily measurable.

There are several types of biomarkers, but three types will de discussed here in the accompaniment of our study, which are as follows:

(a) Biomarkers of DNA damage: There is a sequence of events between the first interaction of a xenobiotic with DNA and consequent mutation: the first stage is the formation of adducts; the next stage may be secondary modifications of DNA, such as strand breakage or an increase in the rate of DNA repair; and the third stage is reached when the structural perturbations in the DNA become fixed and the affected cells often show altered function. One of the most widely used assays to measure chromosomal aberrations is sister chromatid exchange (SCE). Finally, when the cells divide, damage caused by xenobiotics can lead to DNA mutation and consequent alterations in the descent (Walker et al 1996, Shugart 1996).

(b) Biomarkers of gene expression: The development of many tumours related to xenobiotics is associated with the aberrant expression of genes that encode proteins involved in cellular growth. This aberrant expression can involve a quantitative difference (overexpression of the protein) and a qualitative difference (expression of a mutant form of the protein). Although these biomarkers are affected not only by toxic compounds, it is very important to establish potential confounding factors and to assess the sensitivity, specificity and predictive value

of these tests. Biomarkers of gene expression include (Poirier 1997, Chang et al 1994, Brandt-Rauf 1997):

- Growth factors.
- Oncoproteins: (i) growth factor receptors;
  (ii) other oncogene proteins;
  (iii) tumour-suppressor proteins.

It has been reported that, in subjects developing cancer, during the first stages of the disease a significant increase in gene-expression biomarkers related to the specific cancer is seen.

(c) Biomarkers of oxidative damage: Changes in the antioxidant systems and modified macromolecules can serve as biomarkers for a variety of xenobiotics. The protective systems included oxidised glutathione/reduced glutathione, glutathione reductase, catalase, superoxide dismutase and peroxidase activities, ascorbate and α-tocopherol. Macromolecules that may be affected by free-radical damage include lipids, proteins and nucleic acids (Hoffman et al 1989, Di Guilio et al 1969, Hogson and Levi 1994)

The chemical analysis combined with genotoxicity tests revealed mutagens, clastogens and gene convertants in all the investigated ingredients of raw betel nut (RBN) like ARC, tannic acid, catechol. Unfortunately the chewing of RBN continues unabated among the hill tribes of the northeastern region of India. The tribal population of this region chew RBN where ARC is present in higher concentrations (Stich et al 1983). The average age of onset of chewing was 12 years. Thus in the period between incidence of chewing and diagnosis of leukoplakia/tumors, the oral mucosa was exposed for about 28,000 hrs (3.2yrs.) to betel quid extracts. Stich et al (1983) had shown that the saliva of BN and BQ chewers of NE region of India having potent clastogenic activities. Buthionine Sulfoximine (BSO) is a selective and potent inhibitor of GSH synthesis, which inhibits the enzyme γ-glutamyl cysteine synthetase. Since the conjugation of toxic chemicals with GSH *in vivo* is one of the chief methods of their detoxification and

from the recent report that depletion of GSH level enhanced the effect of ARC (Deb and Chatterjee 1998) and gamma radiation (Chatterjee and Chattopadhyay 1998) in mammalian cells, it is of interest in this context to explore the relation between any possible genotoxic effect and the endogenous GSH level.

Thus, on the basis of these background informations, the objectives of the present investigation are:

- The genotoxic effect of RBN-extract in normal and GSH depleted mammalian cells.
- To determine the extent of cytogenetical damages and its relation to endogenous GSH level in raw betel nut chewers.
- To determine the level of p53 protein in betel nut chewers and non-chewers.
- Sensitivity of betel-nut chewers and non-chewers to a mutagen.

CHAPTER I

GENOTOXIC EFFECT OF RAW BETEL NUT IN MAMMALIAN CELLS

Literature Review

Betel-nut (BN) or areca-nut is the hard, edible, endosperm of the palm *Areca Catechu* Linn (Palmaceae) which grows throughout South and South-East Asia and in several Pacific Ocean islands. In most of these regions BN is used as a masticatory either alone or as a component of the betel-quid (BQ) along with leaves of *Piper betle* (Piperaceae), slaked lime and chewing tobacco. The chemical composition and pharmacological actions of BN have been reported and reviewed by several workers (Arjungi 1976, Mazumdar et al 1982, Jeng et al 2001).

BN chewing related oral mucosal lesions are potential hazards to a large population worldwide. It has been estimated that there are about 600 million people who chew BQ regularly throughout their life (Sharan 1996). There are strong indications for a causal association between betel-nut or quid chewing habit and oral mucosal diseases such as leukoplakia, oral submucous fibrosis and oral cancer (IARC 1985, Ko et al 1995). However, the precise mechanisms of oral cancer are still not fully elucidated. Most of the previous studies have focussed on evaluating the mutagenicity, genotoxicity and cytotoxicity of various BQ ingredients using mammalian cells or bacterial testing systems (IARC 1985, Sundqvist et al 1989, Jeng et al 1994, Jeng et al 1999). BN-extract (BNE) can induce DNA strand-breaks, SCEs and micronuclei in various kinds of cells (IARC 1985, Sundqvist et al 1989, Dave et al 1992). This extract is mutagenic in Chinese hamster V79 cells (Shirname et al 1984) and can induce cancerous lesions on the hamster cheek pouch (Ranadive et al 1979). Mutagenicity and genotoxicity of betel alkaloids, polyphenol and tannin fractions have been reported (Jeng et al 2001, Panigrahi and Rao 1986, Azuine and Bhide 1992, Deb and Chatterjee 1998) although there are some reports which tell that BN-polyphenol and tannins are not mutagenic (Nagabhusan and Bhide 1988, Wang et al 1999). The i.p. injection of BNE and areca tannin to Swiss albino mice have been reported to substantially elevate SCEs of bone marrow cells (Panigrahi and

Rao 1986). BNE induces SCE and CAs in cultured Chinese hamster ovary cells (CHO) (Dave et al 1992). The frequency of lymphocytic SCEs was elevated in BN chewers and oral cancer patients in comparison to non-chewer controls (Adhvaryu et al 1991). Such an increase in DNA damage among BN chewers signifies the in vivo genotoxic effects of BN on the non-target tissue and its possible role in increasing the risk of cancers at sites other than the oral cavity.

Arecoline (ARC), the most potent alkaloid of BN, interferes with the biosynthesis of macromolecules. It is a monofunctional alkylating agent as it loses only one of its methyl groups during metabolism (Boyland and Nery 1969) and binds with nucleic acids and protein (Nery 1971). The secondary effect of the binding is the depurination, which may lead to the breakage of the chromosome. ARC also shows other carcinogenic properties such as cysteine-adduct formation (Boyland and Nery 1969, Nery 1971) and metabolization by 1-oxide formation (Nery 1971). It decreases the incorporation of [^{3}H]-thymidine in muscle and kidney tissues and inhibits DNA synthesis, decreases the net RNA level and also decreases the protein content of the tissue (Panigrahi and Rao 1982). Both ARC and aqueous BNE when injected intraperitoneally (i.p) into Swiss mice increased hepatic DNA and RNA content and stimulated DNA and RNA synthesis in liver (Shivapurkar et al 1978, Shivapurkar and Bhide 1979). It has also been shown that ARC was able to inhibit biosynthesis of DNA and proteins of Hep2 cells in vitro in a dose dependent manner and reduce the viability of cells (Wary and Sharan 1991). The cytotoxic and thiol depleting properties of ARC appear to be chemically related to the presence of methyl ester group (Sundqvist et al 1989). ARC was also reported to affect poly-ADP-ribosylation of chromosomal proteins, which lead to alterations in the structural organizations of chromatin (Saikia et al 1995).

The intragastric intubation of aqueous BNE daily, five times a week induces tumors in Swiss albino mice and C17 mice (Bhide et al 1979). The subcutaneous injection of BNE leads to transplantation fibrosarcoma in Swiss albino mice (Ranadive et al 1976). Feeding Swiss albino mice a diet containing unprocessed

BN has been reported to induce mucosal hyperplasia, epithelial atypia, but no marked tumor formation (Rao and Das 1989). Chemical carcinogenesis has been shown to be a multi-step process, including initiation, promotion and progression (Cohen and Ellwein 1991, Hursting et al 1999). Thus, elucidation of the potential roles of BN ingredient in the tumor promotion process becomes an attractive issue.

The study was also performed where mice were chronically exposed to extracts of BN (1mg ml^{-1}) to mimic the constant exposure in humans chewing BN. Panigrahi and Rao (1982,1983) reported that the chronic exposure of mice to ARC results in the enhancement of CAs and SCEs over the control value. BNE was found to be teratogenic in chick embryo and ARC was assumed to be the most potent inducer of teratogencicity (Paul et al 1996). The enhancing effects of dietary administration of BN on carcinogenesis in the liver and upper digestive tract was also observed (Tanaka et al 1983). When ARC or BNE was administered by different means like oral intubation (Bhide et al 1979, 1984) mixed with the diet (Dunham et al 1975) and cheek pouch application (Dunham et al 1975, Rao 1984) in different animals, there was positive induction of tumours in both target (Cheek-pouch, oesophagus and stomach) and non target (lung and liver) tissues. Tumour induction in any tissues was not observed when ARC or BNE was given through i.p. route (IARC 1985, Shivapurkar et al 1980). Therefore it seems that metabolic activation of ARC is needed for final conversion into ultimate carcinogens and it is strongly influenced by physiological conditions and the presence of certain factors (Wenke and Hoffman 1983) which could probably be available in the oral route. It has been reported that ARC-OA induced a higher frequency of CAs, SCEs and a greater delay in the cell cycle progression than ARC-IP (Chatterjee and Deb 1999). Therefore, in the present study mice were chronically exposed to raw betel-nut extract **(RBNE)** orally. Though mouse bone marrow cells is usually not directly exposed to ARC, however, this is an ideal *in vivo* model for assessing individual susceptibility to

the genotoxic effects of mutagenic carcinogens (Department of Health Report 1989). It is true that in vitro tests possess little activation and detoxification capability and since mutagenic activity may be dependent upon specific target site metabolism *in vivo*, the mutagenic activity detected *in vitro* must be evaluated *in vivo* (Ashby 1992). Therefore, we evaluated the RBNE in both in vivo and in vitro systems.

Viable DNA lesions form the initial step in carcinogenesis, and short-term assays like SCEs and CAs form simple and quantifiable markers of DNA damage. And if there is damage to DNA then DNA repair machinery should be activated for repair of the lesions formed. This will lead to arrest in cell cycle. Therefore, in addition to these endpoints, cell cycle kinetics will also be studied in normal and GSH depleted mouse bone marrow cells (BMCs) and human blood lymphocytes (HPBLs). One reason for evaluating structural CAs, that has remained essentially unchanged for many years, is that there is a clear association between chromosome rearrangements and cancer formation (Solomon et al 1991, Rabbits 1994, Mittelman 1994).

The study of chromosomal damage at metaphase allows for the observation of a greater number of division figures and presents a more precise and detailed picture of the effects of any mutagenic / carcinogenic agent than does anapahse or telophase analysis. Any cell population, which under normal conditions or by virtue of stimulated cell growth, can provide sufficient metaphase cells for cytogenetic analysis can be used in the evaluation of aberration induction *in vivo*. BMCs are ideal for this purpose. HPBLs are used as these cells are easily attainable, grow rapidly in tissue culture and large numbers of cells in metaphase can be obtained. In the present study several cytogenetical end-points like CAs, SCEs and cell cycle kinetics have been considered with a hope that they will provide informations relative to the sensitivity of each endpoint as well as to the potential hazard for the population.

Chromosomal Aberrations (CAs)

CA assay has been used as an effective screen for evaluating the possible genotoxic potential of any agent. Chromosome alterations have been studied for nearly a century, and it was immediately appreciated that they are associated with malignancy (Boveri 1902). Numerous *in vitro* and *in vivo* studies have shown that CAs can result from exposure to chemicals and ionizing and non-ionizing radiation. The association between specific cytogenetical alterations and tumorigenesis is strong (Mittelman 1994). The idea of a causal association between CAs and cancer risk is based on the concept that genetic damage in lymphocyte reflects similar damage in cells undergoing carcinogenesis.

Sister chromatid exchanges (SCEs)

SCEs are the cytological manifestation of interchanges between DNA replication products at apparently homologous loci, and involve DNA breakage and reunion (Latt et al 1981). SCEs are produced at or near DNA replication fork, hence called S-phase phenomena but not meiotic event (Wolff et al 1974). SCEs have been commonly employed to evaluate cytogenetic responses to chemical exposure and excellent dose response relationships for hundreds of chemicals have been established in a wide variety of *in vitro* and *in vivo* short term experiments (Tucker et al 1993b). SCEs are efficiently induced by those substances that form covalent adducts to the DNA or otherwise interfere with DNA metabolism and repair (Perry 1980, Natarajan et al 1981, Carrano and Thompson 1982) and is a means by which the cell copes with DNA damage (Evans 1977, Shafer 1982). Increased SCE is an indirect measurement of mutation resulting from DNA damage and of mitotic rearrangement of genetic material. Consequently, SCE analysis must be relevant in the evaluation of genetic hazards (Beneditt and Beneditt 1973).

Cell cycle

Chemical damage to DNA is itself not a mutagenic event, but if unrepaired can be converted to a mutagenic event during the process of DNA replication (Bertram 2001). Because DNA synthesis itself is a tightly controlled, highly

coordinated process, delays in progression through S-phase as a consequence of DNA damage or insufficient availability of protein or DNA precursors frequently result in cell death, chromosomal abnormalities or mutations. Since these two latter events are intimately associated with carcinogenesis, it is not surprising that many of the genes found to be damaged in cancer cells have actions that relate to cell cycle checkpoint control. The genome should be damaged free before the onset of replication. To ensure that a cell has all the nutritional support for the synthesis of the new strands of DNA and the protein matrix to allow packaging of the newly synthesized DNA into chromatin, mammalian cells have devised elaborate checkpoints to prevent premature entry into the division cycle. The most significant checkpoint occurs in late G1, approximately 4 hours prior to the cell's entry into the S-phase. Activation of this checkpoint control in response to DNA damage delays entry into S-phase and provides the cell the time necessary for repair. At G1-stage the stability of p53 protein will be increased in the cells if it gets exposed to the mutagenic agents and thereby induce the expression of genes related to cell cycle arrest (Smith et al 1995, Kastan et al 1991). Mutations in the p53 gene are the most common genetic alterations in human cancers, illustrating the critical importance of cell cycle regulation in the life of multicellular organisms.

Employing the FPG-technique (Goto et al 1975) cell cycle delay can be measured in terms of reduction in the frequency of 2^{nd} and subsequent division metaphases, and corresponding increase in the first cycle metaphases, at a given time following the treatment. In this study, cell cycle kinetics *in vivo* was determined by mitotic index (MI) and in HPBLs by using FPG-technique.

The North-Eastern Indian variety of BN is raw, wet and unprocessed consumed with betel-leaf and slaked lime. The constituents of this nut shows higher alkaloids, polyphenol and tannins as compared to the dried one (Sharan 1996). It has been reported that in the raw betel nut the quantity of alkaloids, polyphenols, tannins is more than in the cured ones (Raghavan and Baruah 1958). From this

North-Eastern region of India, a higher frequency of occurrence of micronucleated cells has been reported from buccal mucosa of people who chew RBN (Stich et al 1982, Stich et al 1983). So, for our study, raw betel nut (RBN) was used for all experiments both *in vivo* and *in vitro*.

Glutathione (GSH) consists of γ-glutamic acid, cysteine and glycine is the most prevalent non-protein intracellular thiol present in high concentrations (0.5 to 10nm) in almost every cell. It is found throughout the cell, with the bulk in the cytoplasm, subcellular particles, such as the nucleus and mitochondria having smaller amounts (Biaglow and Tuttle 1993). It is a major component of several intracellular anti-free radical enzymes like GSH-peroxidase and GSH- reductase. GSH is probably the most important cellular antioxidant. Interestingly, Fahey and Sundqvist (1991) found strong evidence for an evolutionary link between GSH and aerobic eukaryotic metabolism, the findings indicate that GSH evolved as a molecule that protects cells against oxygen toxicity. Cells that are deprived of GSH typically suffer several oxidative damage associated with mitochondrial degeneration. Human deficient in GSH may exhibit increased tendency to hemolysis, cataracts, and central nervous system abnormalities. In one condition (glutathione synthetase deficiency) there is a secondary metabolic acidosis, often life threatening, due to overproduction of 5-oxoproline. The cells of these patients showed higher radiosensitivity in hypoxic condition (Revesz et al 1984). The most useful approach to deplete the level of endogenous GSH is the treatment with buthionine sulfoximine (BSO), a specific inhibitors of γ-glutamylcysteine synthetase, the enzyme that catalyzes the step of GSH synthesis (Griffith and Meister 1978, Griffith et al 1979). BSO is a potent and selective inhibitor of GSH synthesis that is highly effective both *in vivo* and *in vitro* without showing any side-effect (Griffith and Meister 1978, Griffith et al 1979). Depletion of GSH by treatment with BSO sensitizes the cells to the toxic effects of heavy metals (Singhal et al 1987, Naganuma et al 1990), nitrogen mustard (Suzukake et al 1982, Suzukake et al 1983), radiation and cisplatin (Edgren and Revesz 1987,

(Anderson et al 1990, Chattopadhyay et al 1999), cyclophosphamide (Ishikawa et al 1989a, Ishikawa et al 1989b), compounds that produce oxidative cytolysis (Arrick et al 1982) and others (Perez et al 1990), mitomycinC (Dev-Giri and Chatterjee 1998). GSH deficiency leads to oxidative stress in many tissues (Meister 1991). Mitochondrial and associated cell damage is found in mice treated with BSO. Several tissues of adult mice are affected by administration of BSO, but in new born rats and guinea pigs more extensive damage is found and there is early mortality due to multi organ failure (Martensson et al 1991). Treatment of peripheral blood mononuclear cells with BSO was found to markedly inhibit their proliferation (Meister 1983). Human lymphoid cells depleted of GSH exhibited increased sensitivity to radiation (Dethmers and Meister 1981). Tumour cells depleted of GSH by BSO exhibit increased susceptibility to cytolysis by reactive oxygen intermediates (Arrick et al 1982). Also it has been reported that depletion of GSH by BSO in tumour cells indeed increase the cytotoxicity of a variety of anticancer drugs (Green et al 1984, Lee et al 1986).

Following a single dose of 2.5 nmol kg^{-1} BSO, the GSH contents of the various normal tissues were depleted in a time dependent manner. Kidney and liver showed the most rapid rates of depletion, with GSH levels reaching nadirs by ~5h. Intermediate rates of depletion were seen in the lung and bone marrow with nadirs at 4-16h and 8-12h respectively. The heart was depleted the most slowly with a nadir in GSH at 24-72h. The extent of GSH depletion following a single dose of BSO also differed significantly between tissues. The most severe depletion occurred in the liver, kidney and bone marrow, the heart and the lung showed intermediate depletion, and RBCs showed the least depletion. Recovery to pretreatment values, was most rapid for the liver (16h) followed by the kidney (30h), the lung (32h) and bone marrow (72h). Recovery was extremely slow for the heart (>96h) and RBCs (>72h). For lung, and in particular the liver and kidneys a pronounced 'overshoot' in GSH levels occurred during recovery i.e. GSH concentration rose significantly above those for untreated controls (Lee et

al 1987). Earlier studies have also shown that 10h of 200 mg kg^{-1} BSO treatment in mouse bone marrow depletes the concentration of GSH to 54% of the control value (Chattopadhyay et al 1999).

As GSH plays an important role in cellular defense mechanisms (Deleve and Kaplowitz 1991), the genotoxicity of RBN in relation to cellular GSH level was investigated. And it is well known that GSH protects cells against radiation (Chatterjee and Jacob-Raman 1986) and various toxic effects of xenobiotics (Shaw and Chou 1986, Dev-Giri and Chatterjee 1998, Syng-ai and Chatterjee 2002) but not against radiomimetic drugs like bleomycin (Chatterjee et al 1989, Chattopadhyay et al 1997). The ARC induced CA in mice and this was enhanced by BSO (Deb and Chatterjee 1998) and the genotoxic effect of ARC was reduced when it administered with N-acetyl-L-cysteine (NAC) (Chatterjee and Deb 1999).

The p53 gene, located on chromosome 17, encoding a 53-kd nuclear phosphoprotein, is part of the cell's emergency team that is called upon following cellular insult. It is known to regulate cell growth and proliferation (Isobe et al 1986, Baker et al 1990, Kuerbitz et al 1992) and it remains the most frequently targets of genetic alteration identified in human cancers. Loss of p53 function, most commonly through point mutations within one of the evolutionary conserved domains, occurs in approximately half of most major cancers, and the essential role played by p53 in tumour suppression is illustrated by the rate of malignancies in mice lacking functional p53. Interestingly these mice develop more or less normally, suggesting that p53, which rather unusually is not a member of a larger protein family plays no essential role in regulation of the normal cell cycle in most cells. Rather, the principal function of p53 appears to be in mediating a response to DNA damage, thereby preventing accumulation of potentially oncogenic mutations and genomic instability (Lane 1992).

Normally in a cell, the p53 protein is kept at a low concentration by its relatively short half-life (~20 min) in non-stressed cells. In some cells, p53 probably also

exists in a latent form, inactive for transcription. Several types of DNA damage can activate p53, including double strand breaks in DNA produced by γ-irradiation and the presence of DNA repair intermediates after UV-irradiation or chemical damage to DNA. Many post-translational modifications of p53, such as phosphorylation, dephosphorylation, acetylation and ribosylation, have been shown to occur following stress. The cellular response to genotoxic agents initiates a rapid and substantial increase in the total p53 levels, achieved in part by the stabilization of the normally rapidly degraded protein. Activation of p53 in this way leads ultimately to the suppression of cell growth, a function which is also evident following reintroduction of wild-type p53 into tumour cells lacking normal p53.

p53 mutations can give rise to conformationally altered, functionally defective proteins which have a longer half-life than their wild-type form. As a result, the p53 mutant protein can be detected by immunohistochemical means (Iggo et al 1990). However, immunopositivity for p53 is not always associated with gene mutations (Xu et al 1994). The wild type p53 may become detectable by immunohistochemistry due to stabilization of the gene product by binding to p53-associated proteins (Battifora 1994)

The p53 gene and its protein product have become the centre of intensive study ever since it became clear that slightly more than 50% of human cancers contain mutations in this gene. Overexpression of p53 has been found in 40-70% of head and neck carcinomas and in one half of the non-malignant epithelia adjacent to positive tumours (Warnakulasuriya and Johnson 1992, Field et al 1991, Shin et al 1994). Exposure to radiation leads to an increase in the levels of proteins that derives from an alteration in its half-life as a result of post translational modifications (Levine 1997, Ko and Prives 1996). Similar observations were made with exposures to BLM and other chemotherapeutic drugs (Lu and Lane 1993). So, it was of interest to determine the levels of p53 protein in mouse BMcs after feeding orally with the RBNE.

Therefore, the present study was undertaken to determine the genotoxic effect of both RBN water and acetic-acid extract in mammalian cells with respect to endogenous GSH level. In fact reports are not adequate regarding the effect of BNE on cell cycle kinetics and its relationship with BNE-induced DNA damages. We have also made an attempt to see the level of p53 protein in mouse in vivo after feeding the BN aqueous extract for 1,5 and 15 days.

Materials and methods

Materials

Mouse bone marrow cells (BMCs): Inbred Swiss albino mice of 6-8 weeks, weighing about 20-25 gms (mostly males) were used for all *in vivo* experiments (2n=40). Mice were maintained in communal cages, with sterile bed under controlled temperature ($20^0C \pm 2$) and lighting condition (12h light and 12h dark). Standard animal feed (NMC oil mills Ltd., Pune India) and water was provided *ad libitum*. For all types of experiments BMCs were isolated just before use.

Human peripheral blood lymphocytes (HPBLs): For all *in vitro* experiments, HPBLs were used. Blood was collected from healthy young male donors (25-30 years). For culture whole blood was used. For tGSH measurement from HPBLs, first of all, lymphocytes were separated from the heparinised whole blood on a Ficoll-hypaque (FH) density gradient by the method of Boyum (1968).

PREPARATION OF EXTRACTS OF RAW BETEL NUT (RBNE)

Two types of extracts were chosen for this study:

- Aqueous extract of BN (AEBN): This was chosen because saliva is water based and in natural conditions of human consumption the BN components are extracted in it.
- Acetic acid extract of BN (AAEBN): AAEBN extract was prepared to mimic the condition of extraction in acid pH as in buccal cavity and digestive system in humans.

Unprocessed, raw BNs were purchased from the local market. After shelling the fibrous coats, 100g of BN are ground and suspended separately in 125 ml of (a)

distilled water for aqueous extract of BN (AEBN) or (b) 1% acetic acid for acetic acid extract of BN (AAEBN) and mixed thoroughly to give smooth paste. This was kept overnight at 4-8°C. Next day it was stirred for 3h at 37°C and the extract was collected by centrifugation. This extraction procedure was repeated once more by adding 125ml of water to the residue. Both the extracts were pooled which represents 100gm BN in 250ml distilled water, was filtered through 0.22μm-porosity millipore filter, kept at –80°C for complete freezing. The filtrate was lyophilized in a Secfroid Lyolab BII Lyophilizer. The lyophilized mass was kept at 4°C until use. The extract contained 0.9g/100g water-extractable material.

Reagents for glutathione estimation:

- AEBN / AAEBN: For *in vivo* mice were administered orally with a dose of 200μg ml^{-1} and it was estimated that each mouse consumed 1mg of extract in a day. For *in vitro* a working solution of 1000μg ml^{-1} was prepared in distilled water just before use and the different doses were added into freshly collected blood.
- DL-Buthionine- (S,R)-Sulfoximine (BSO, Sigma, USA): This is a potent inhibitor of the enzyme γ-glutamyl cysteine synthetase of GSH pathway. BSO was freshly prepared in double distilled water.
- Ficoll-hypaque (Sigma, USA) 1.077 gm ml^{-1}
- Reduced Glutathione (GSH; sigma, USA): Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is a tripeptide but is not derived from protein. Here, a glutamic acid residue is joined in an unusual peptide linkage involving its γ-COOH rather than the α-COOH group with cysteine. This thiol has been shown to protect biological tissues by scavenging the primary radicals produced by radiolysis of water. Different concentrations of this tripeptide were used for standard curve.
- 5-sulfosalicyclic acid (5-SSA) (Merck, India), dehydrate 5% and 10%.
- Ethyldinitrilo tetra acetic acid (EDTA) (Merck, India).

- Potassium dihydrogen phosphate (KH_2PO_4, S.D. fine chem Pvt. Ltd. Boisar) prepared in distilled water

  Solution A 0.1 M
- Dipotassium hydrogen phosphate (K_2HPO_4, S.D fine chem Pvt. Ltd., Boisar) prepared in distilled water

  Solution B 0.1 M

  39 ml of solution A was mixed with 61 ml of solution B and pH adjusted to 7.0. EDTA was added to achieve a final concentration of 1mM EDTA phosphate buffer.
- Sodium bicarbonate 0.5% ($NaHCO_3$, Merck, India)
- 5-5'-dithiobis-2-nitrobenzioc acid (Ellman's reagent, DTNB; Roche molecular Biochemicals ,Germany)

  1.5 mg of DTNB was dissolved in 0.5% $NaHCO_3$ (prepared fresh at the time use).
- Nicotinamide Adenine Dinucleotide Phosphate tetra sodium salt (NADPH, SRL, India).

  4 mg of NADPH was dissolved in 0.5% $NaHCO_3$ (prepared fresh at the time of use).
- Glutathione Reductase from yeast (GR, Roche molecular biochemicals, Germany).

  A solution of 6 units/ml was made in 0.1 M phosphate buffer, pH 7.0 just before use and kept in ice. Freezing was avoided as it leads to denaturation and loss of enzyme activity.
- Hydrochloric acid (10mM).

Treatment:

AEBN: For GSH estimation, only AEBN was used. Mice were fed orally with drinking water (200$\mu g\ ml^{-1}$) for 1 and 5 days and it was estimated that each

mouse consumed 1 mg of extract in a day. For *in vitro*, 250µg ml^{-1} of AEBN / AAEBN was added 2 hours prior to estimation.

BSO: BSO at a dose of 50 mg kg^{-1} b.wt. was injected intraperitoneally in mice for 1 and 5 days. In case of 5 days treatment, BSO was given every 60 hours. Mice were sacrificed after 1 and 5 days. For *in vitro*, BSO (5mM) was added to freshly collected heparinized blood for 3h prior to estimation.

***Principle of GSH estimation*:** The recyclic assay or kinetic assay used in this method utilizes the continuous glutathine reductase (GR) catalyzed reduction of the sulphydryl reagent 5,5'-dithiobis-2-nitrobenzioc acid (DTNB, Ellman's reagent) to the chromophoric product 5-thio-2-nitrobenzoic acid (TNB) and glutathione disulfide (GSSG). GSSG is reduced to GSH by the action of GR and NADPH reduced sodium salt. The chromophore is monitored spectrophotometrically at 412nm and is proportional to the sum of GSH and GSSG present.

$$2GSH + DTNB \rightarrow GSSG + TNB$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Quantitation is achieved by comparison with a standard curve of known GSH concentration. The procedure is highly specific as it utilizes enzymatically catalyzed conversion to GSSG to GSH.

Sample Processing:

IN VIVO: Mice were sacrificed by cervical dislocation and BMCs from femur were flushed into ice-cold EDTA-phosphate buffer. They were mixed thoroughly, the volume was made to 1 ml and cells were counted in a hemocytometer.

IN VITRO: The blood lymphocytes were first separated from the heparinised whole blood on a Ficoll-hypaque / histopaque density gradient by the method of Boyum (1968).

The sample (blood) solution is layered on a preformed step gradient in a centrifuge with the denser solution at the bottom. Histopaque is a solution of polysucrose and sodium diatrizoate adjusted to a density of 1.077g/ml. During

centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sediment whereas lymphocytes and other mononuclear cells remain at the plasma histopaque interface. In order to achieve separation, the density of sample particles must be greater than the density at any specific position along the gradient column.

Procedure:

1. Blood was drawn by venipuncture and collected in vials containing 25 IU of heparin and diluted 1:1 with sterilized RPMI 1640 medium.
2. In a centrifuge tube, 2ml of histopaque was taken, then 2 ml of the diluted blood was gently layered on top of 2 ml of the separation medium.
3. The tubes were centrifuged at 1200 rpm for 40 min.
4. The ring at the interface of histopaque and upper plasma layer is the lymphocyte ring.
5. The lymphocyte ring was sucked out with the help of a syringe and transferred to another tube.

Sample processing :

1. 50µl of undiluted sample (BMCs/ HPBLs) suspension was transferred into a micro centrifuge tube containing 150µl of 10 mM HCl.

   Acid treatment reduces oxidation of GSH to GSSG and to mixed disulfides and also inactivates γ-glutamyl transpeptidase, which catalyzes the following reactions that decrease the levels of both GSH and GSSG.

   $GSH+aa \Leftrightarrow \gamma$ Glu-aa + CySH-Gly (Transpeptidation)

   $GSH + H_2O \rightarrow$ Glu =CySH – Gly (Hydrolysis)

   $GSH + GSH \Leftrightarrow \gamma$Glu-GSH + CySH-Gly (Autotranspeptidation)
2. Cells were lysed by alternate freezing and thawing at least three times at 10°C and room temperature for 10 minutes respectively and centrifuged in a microfuge at 10,000 rpm for 5min.
3. The supernatant was deproteinized using 100µl of ice cold 105 5-SSA with intermittent shaking.

4. The tubes were kept in ice for 10-15 min and the acid precipitable proteins were removed by centrifuging at 10,000 rpm at 4°C for 15 min in a Beckman J2-HS centrifuge. The supernatant was immediately used for GSH determination.

Advantages of the processing method:

1. Acid precipitation of samples maintains proper thiol-disulfide redox status (GSH oxidizes rapidly at pH values above 7.0). Acidification allows for the precipitation of proteins and ensures cell lysis and subsequent release of free thiols and disulfides. It also avoids GGT catalyzed degradation of GSH.
2. 5-SSA was preferable for deproteinization because TCA, perchloric and meta-phosphoric acids do not maintain true GSH: GSSH ratios and also these acids interfere with subsequent enzymatic reactions.
3. Chelating agents such as EDTA were used in the sample buffer so as to prevent iron-mediated formation of peroxides in the presence of oxygen.

Total GSH was estimated according to the method of Theodorus et al (1981).

1. To 1 ml of the buffer taken in a cuvette 50µl of sample suspension, 50µl of NADPH, 20µl of DTNB and 20µl of GR were added. GR was added to initiate the assay.
2. The contents were mixed and the formation of TNB was followed continuously with a record for a total of 5 min. at 412 nm in a Beckman DU-640 spectrophotometer.
3. The amount of GSH was calculated from a standard curve where the GSH equivalents were plotted against the rate of change of absorbency at 412 nm.

Standard curve was prepared from a stock solution of 10mM GSH (30.7 mg ml^{-1}) in 5-SSA diluted to 100-1000 nmol GSH ml^{-1}. A sample blank lacking GSH was used to determine the background rate.

The values were reported in GSH equivalents as µmol / $1x10^6$ cells in all cases.

Reagents for in vivo and in vitro (culture) cytogenetic study:

- AEBN/AAEBN

- BSO
- 5-bromo-2-deoxyuridine powder (BUdR, Sigma, USA): This was used only for *in vitro* experiments. BudR was dissolved in RPMI 1640 culture medium and added to each culture tube at a concentration of 6 μg ml^{-1}.
- Phytohaemmagglutinin M (Gibco,USA)
- RPMI 1640 (Hyclone, USA) with penicillin and streptomycin (Hyclone, USA)
- New born calf serum (Hyclone, USA)
- Bis- benzimide (Hoechst 33258, Sigma, USA.)
  50μgml^{-1} working solution was made in double distilled water.
- Colcemid (Gibco, USA).
- Giemsa, 3% (Merck,Germany)

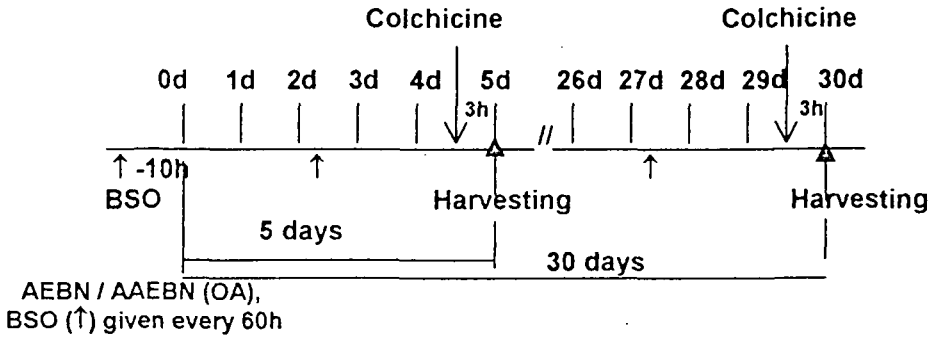
All other chemicals were of analytical grade.

Treatment:

AEBN/AAEBN: For *in vivo* study both AEBN and AAEBN (200μg ml^{-1}) were administered ad libitum in the drinking water. Such oral administration was continued for 5 and 30 days. For *in vitro*, two concentrations of both types of extracts were used viz. 100 and 250 μg ml^{-1} of blood and cells were fixed at 72h.

BSO: BSO was injected i.p. in mice 10h before extract administration. Thereafter for both 5 and 30 days treatment, subsequent treatment of BSO was given every 60 hours after they were exposed to AEBN or AAEBN mixed water. For *in vitro* system, BSO was added in HPBLs at a concentration of 5 mM 3 hours prior to extract treatment.

The treatment procedure for *in vivo* study is shown below.



Culture procedure

Cultures were set up in medium containing RPMI 1640 with antibiotics and supplemented with 10% heat inactivated new born calf serum. For each culture 1 ml blood was used. In order to stimulate the G_o lymphocytes 0.2ml of phytohaemagglutinin M was added in each culture. To obtain differential staining, 6μg ml^{-1} 5-bromodeoxyuridine was added to each culture. All cultures were incubated at 37°C. After about every 12h cultures were gently shaken. Cultures were harvested at 72h to analyze the effect of BNE and colcemid (0.01 μg ml^{-1}) was added 3h prior to that. All experiments were repeated at least three times with a few exceptions.

Preparation of metaphases

At the end of 5^{th} and 30^{th} day, mice were sacrificed by cervical dislocation with 3h prior treatment of colchicine (10 mg kg^{-1} bw) in each animal. The femur bones were dissected out and the BMCs were obtained by flushing out with 2 ml prewarmed (37°C) 0.075M KCl with the help of a hypodermic syringe and a 26-gauge needle. A single cell suspension was made in hypotonic solution and incubated for 15 min at 37°C. The cells were centrifuged at 1200 rpm for 5 min. and fixed in two changes of fixative for 30 and 10 min respectively, resuspended in 1ml of fixative and dropped onto a grease free chilled slide and flame dried.

In case of *in vitro* experiments, colcemid at a concentration of 0.01 μg ml^{-1} was added 3h prior to harvesting. Hypotonic treatment was done for 20 min and cells were fixed and slides were prepared according to the flame drying method as stated above.

Differential staining

Differential staining of sister chromatids were carried out by the method of Goto et al (1975). Slides were treated for 10 min. with Hoechst 33258 (50μg ml^{-1}) at room temperature in the dark. The slides were then rinsed with distilled water, mounted in 2XSSC (NaCl-Na-citrate, pH 6.8) and kept in sunlight for 30-40 min.

depending upon the intensity of light. After rinsing in double distilled water, slides were stained in 3% Giemsa for 3-4 min, air dried and mounted in DPX.

Scoring and statistical analysis

Slides were coded at random. All metaphases were scored for mouse BMCs whereas for human PBLs metaphases were categorised as in first, second and subsequent division cycles based on their differential staining patterns for in vitro studies.

For mouse BMCs all CAs were scored from well spread metaphases and for mitotic index number of metaphases were scored out of total number of cells observed in one focus. For humans PBLs aberrations were scored from first cycle metaphases (M1) only. For both the cases the aberrations were scored mostly as chromatid breaks. Total numbers of normal as well as aberrant metaphases were scored.

For scoring cell cycle kinetics (*in vitro*), metaphases were categorised as 1st, 2nd and subsequent division cycles based on their differential pattern. The cell cycle data were presented as average generation time (AGT) which is a ratio of BUdR duration (H) and replicative index (R.I), where R.I. = 1XM1 +2XM2 +3XM3 / total number of cells. The BudR duration in the present study was 72 hrs.

For *in vitro* studies, SCEs were scored from 2nd cycle metaphases, at least 25 (with a few exceptions) well spread second cycle were considered for SCE scoring. Metaphase cells with differentially stained sister chromatid from culture were studied for evidence of SCEs. Data were subjected to parametric statistical analysis. To compare the effects of BNE with BSO on the intracellular distribution of SCEs within individual group, the dispersion coefficient H (Snedecor and Cochran, 1967) that is the ratio of the sample variance to the sample mean (Margolin et al 1986) was analysed. SCEs, AGT and M.I will be evaluated using the student's t-test while M1% by 2x2 contingency test. Data were subjected to statistical analysis using the 2x2 contingency test for the frequency of aberrant

metaphases and simple χ^2- test for aberration. The actual observed data were used during statistical analysis.

Reagents for Western blot analysis

- AEBN
- RIPA buffer (Radio Immuno Precipitation Assay) buffer.

1%(w/v) Nonidet P-40 (NP-40) (Sigma, USA)

1%(w/v) sodium deoxycholate (Sigma, USA)

0.1%(w/v) SDS (Sigma, USA)

0.15M NaCl (Merck, India)

0.01M sodium phosphate, pH 7.2 (Merck, India)

2mM EDTA (Sigma, USA)

50mM sodium fluoride (Sigma, USA)

100U/ml aprotinin (trasylol, Pentex / Miles) (Sigma, USA)

- DNAse (Stratagene, USA)
- Genei protein estimation kit by bicinchoninic acid (BCA) method (Bangalore Genie, India)
- SDS-polyacrylamide gel kit (Bangalore Genie, India)
- 10% SDS resolving gel, ready-mix for SDS- PAGE (Bangalore Genie, India)
- 5% SDS stacking gel, ready-mix for SDS-PAGE (Bangalore Genie, India)
- Ammonium persulphate (For SDS –PAGE) (Bangalore Genie, India)
- Tris-glycine buffer (10X supplied, converted to 1X before use) (Bangalore Genie, India)
- Sample buffer (5X concentrate) or gel loading buffer has a blue dye bromophenol blue (Bangalore Genie, India)
- Transfer buffer solution: 40 ml transfer buffer +200 ml methanol + rest water to make a volume of 1lt.

Preparation of transfer buffer: Tris Base (Merck, India) - 18.2g, Glycine (Merck, India)- 90.0g,distilled water-500ml. The pH of the buffer is 8.3

- Blocking buffer: TBST with 5% Non fat dried milk (NFDM), 10X

TBST: 1M Tris HCl (pH 7.4, SRL, India)-3.152g, 5M NaCl (Merck, India)-7.5972g. Mix these two that is Tris HCl and NaCl, Tween 20 added (Bangalore Genie, India) and volume made to 1 lt. with dw.

To prepare working 1X TBST, add 90 ml distilled water to 10 ml of 10X TBST

- NFDM: 1g in 20 ml of 1XTBST
- Primary Antibody: p53-Abs (DO7 + Bp53-12) (Neomarker, Fremont,USA)
- Secondary Antibody: rabbit Antimouse IgG-ALP conjugate (Bangalore Genie, India)
- Substrate: BCIP/NBT, substrate for alkaline phosphatase (Bangalore Genie, India)

  BCIP: 5-bromo-4-chloro-3-indolyl phosphate

  NBT: Nitroblue tetrazolium.
- β-actin (anti-actin ACTNO5, Neomarkers, Fremont, USA) 1:5000 dilutions.

Treatment:

AEBN: Mice were fed orally with AEBN in drinking water for 1, 5, 15 days. Mice were sacrificed and BMCs were isolated.

Protein Extraction:

(1) The BMCs were taken in a centrifuge tube and washed twice in PBS by centrifuging at 1500 rpm for 5min.

(2) Supernatant was discarded thoroughly. 200μl of RIPA buffer was added to the pellets, vortexed and kept in ice.

(3) 5μl DNAse was added to each sample (working solution 1U/μl, DNAse:H_2O=1:9)

(4) The samples were kept in ice for 30 min. and then kept at –80^{0}C.

Estimation of protein:

(1) Eppendorf tubes with samples were taken, kept in ice, vortexed well.

(2) 10μl sample + 90μl H_2O (distilled water) was taken in a centrifuge tube.

(3) Preparation of BCA reagent:

For 20 ml: 19.6ml BCA solution + 0.4ml $CuSO_4$

For 25 ml: 24.5ml BCA solution +0.5ml $CuSO_4$

(4) For sample, 2ml of the prepared BCA reagent was added to the centrifuge tube containing 10µl sample +90µl H_2O. This will be measured against the standard. The standard curve was plotted using different concentrations of BSA (10-160µg).

(5) The optical density of the samples was measured at 562nm by a spectrophotometer (Beckman, DU 640).

The amount of protein measured was converted into µg/µl.

Immunoblotting: Equal amounts of protein (40µg/lane) were separated by SDS PAGE using 10% polyacrylamide resolving gel and a 5% stacking gel at 50V constant voltage for 2hours. The proteins were transferred onto a 0.45 µm nitrocellulose membrane at 50V constant voltages for 7 hours. The membranes were blocked at room temperature in TBST with 5% (w/v) NFDM and incubated with primary Ab (1:1000 dilution) for 1h. After washing thrice (10 min each) in TBST with 5% milk, membranes were incubated with secondary Ab conjugated to Alkaline Phosphatase for 1h (1:2000 dilution) by gentle shaking which catalyses a chromogenic reaction. A primary antibody for β-actin expression was also used for sample loading. The substrate BCIP/NBT (for alkaline phosphatase) was added which is converted in situ into a dense blue precipitate by immunolocalized alkaline phosphate marking the bands. The bands were visible in 10-15 min.

Results

RBNE induced CAs and the frequency of mitotic index (MI) in mouse BMCs and CAs, SCEs and the M1 in human PBLs were studied as positive controls to BSO + BNE treated samples and the data are presented in tabular form. Negative control data are also shown. CAs are mainly of chromatid break type.

The CAs induced by RBNE in mouse BMCs and Human PBLs are shown in figures 1.1, 1.2. Figure 1.3 shows the Heochst-sunlight-Giemsa differential

staining pattern of chromosomes in Human PBLs grown in the presence of BudR.

IN VIVO

(a) Levels of tGSH: The data for tGSH concentration in mouse bone marrow cells is given in Table 1.1, figure 1.4. In untreated group, the concentration of tGSH range from 2.00-4.00 μmol per 10^{-6} cells with an average of 2.95±0.24 μmol per 10^{-6} cells. In case of one day BSO treatment, the GSH level depleted significantly. The mean value was 1.49±0.11 μmol per 10^{-6} cells which means 49% of the control value. In 5 days BSO treatment, there was even more depletion with the mean value of 1.09±0.22 μmol per 10^{-6} cells which means there was depletion of 63%. The concentration of GSH was reduced to 24 and 54% of the control value after 1 and 5 days treatment with AEBN alone.

(b) Chromosomal Aberrations (CAs): Table 1.2, Figure 1.5 shows that both the aqueous and acetic-acid extract induced similar level of CAs and the presence of BSO increased the frequency of aberrant metaphases marginally. It is clear from the data that 30 days treatment of these extracts induced higher frequency of CAs than 5 days treatment. However, the BSO alone increased the frequency of spontaneous aberrations. The aberrations were mainly chromatid break type and BNE failed to induce any exchange aberrations.

(c) Cell cycle kinetics: Table 1.3, figure 1.6 shows the frequency of M.I. and indicate that both the extracts induced significant delay in cell kinetics after 30 days of treatment, however a positive tendency of delay was observed after 5 days treatment also. Presence of BSO further increased the delay in cell cycle progression which was induced by both the extracts after 30 days of treatment. Although the basic cell cycle progression varied considerably among individuals in each group, the induction of delay by the RBNE was very clear. It is also worth mentioning that from the range of delay induced by both the extracts AAEBN induced slightly higher delay in cell kinetics than AEBN. In contrast to the delay

induction by BNE alone in normal mice, BSO alone showed low M.I. values indicating a positive tendency of induction of delay in cell progression.

(d) Levels of p53 protein: Figure 1.11 shows the level of p53 protein in two experiments. In both the experiments the level of p53 protein was higher in 15 days AEBN-treated samples than 1 and 5 days treated samples.

IN VITRO:

(a) Levels of tGSH: Result of tGSH estimated in human PBLs is shown in Table 1.4, figure 1.7. In HPBLs, the levels of tGSH dropped by 84% after 3 hrs of BSO treatment from 5.27μmol/10^6 cells to 0.86μmol/10^6. However the level of tGSH increased significantly after AEBN and AAEBN treatment. An increase of 64% from 5.27 to 8.66μmol/10^6 was observed with AEBN, with AAEBN the increase was 50% from 5.27 to 7.92μmol/10^6.

(b) Chromosomal Aberrations (CAs):

AEBN: Table 1.5, figure 1.8 shows the dose dependent increase in the frequency of aberrant metaphases by AEBN. Treatment of BSO before AEBN treatment failed to increase the frequency of aberrant metaphases significantly. The aberrations observed were mostly chromatid break type. BSO alone did not induce any kind of CAs since no change was observed from the frequency of spontaneous aberrations. From the pooled data (shown in table 1.6) the dose dependent increase in aberrant metaphases is clear.

AAEBN: Table 1.7, figure 1.8 shows the frequency of aberrant metaphases by AAEBN. In contrast to AEBN, both the concentrations of AAEBN showed similar level of induction of aberrant metaphases. Here also, treatment of BSO before AAEBN treatment failed to show any appreciable change in aberrant metaphases. From the pooled data (shown in table 1.8) it is clear that both the concentrations of AAEBN induced significant levels of aberrations which is not dose-dependent.

(c) Sister Chromatid Exchanges (SCEs):

***AEBN*:** Table 1.9, figure 1.10 presents data of SCEs induction by AEBN. The dose dependent increase in SCEs by AEBN is clear. Prior treatment with BSO increased the frequency of AEBN induced SCEs. BSO alone did not induce significant SCEs with respect to control values. The dispersion analysis indicated that the distribution of SCEs in AEBN treated with and without BSO did not deviate from Poisson distribution. From the distribution of SCEs per cell, AEBN treatment yielded more cells having 7 or more SCEs with respect to control. In the presence of BSO along with AEBN, the cells having 11 or more SCEs per cell were increased substantially in most of the experiments. The distribution of SCEs per cell is clear from the pooled data as shown in table 1.10.

AAEBN: The data for SCE induction by AAEBN is shown in table 1.11, figure 1.10. AAEBN increased the frequency of SCEs in most cultures and unlike AEBN, the induction of SCEs was not dose-dependent. This is evident from the pooled data as shown in table 1.12. BSO pretreatment increased the frequency of AAEBN induced SCEs. The dispersion analysis indicated that the distribution of SCEs in AAEBN treated with and without BSO did not deviate from Poisson distribution. Here also, regarding the distribution of SCEs per cell, AAEBN treatment yielded more cells having 7 or more SCEs per cell with respect to control. In the presence of BSO along with AAEBN, the cells having 11 or more SCEs per cell were increased substantially in most of the experiments.

The frequency of SCEs in untreated controls did not differ significantly from each other and the dispersion of SCEs was consistent with a Poisson model for control and all other treated samples.

(d) Cell cycle kinetics:

AEBN: The data for cell cycle kinetics is shown in table 1.5, figure 1.9. There was dose dependent increase in the percentage of M1 cells by AEBN. This is clear from the pooled data (table 1.6) where we see that M1% cells significantly increased by both the doses of AEBN which was increased further with BSO.

Dose dependent increase in AGT value after AEBN treatment was seen in most experiments. Treatment of BSO before AEBN treatment increased the value of AGT.

AAEBN: Table 1.7, figure 1.9 shows the data for cell cycle kinetics by AAEBN. In contrast to AEBN, both the concentrations of AAEBN interestingly reduced the AGT value and the percentage of M1 cells. However treatment of BSO before AAEBN treatment increased the AGT value and also the M1 % cells. The effect of AAEBN on cell kinetics is clear from the pooled data as shown in table 1.8. BSO alone also failed to increase the frequency of M1 cells.

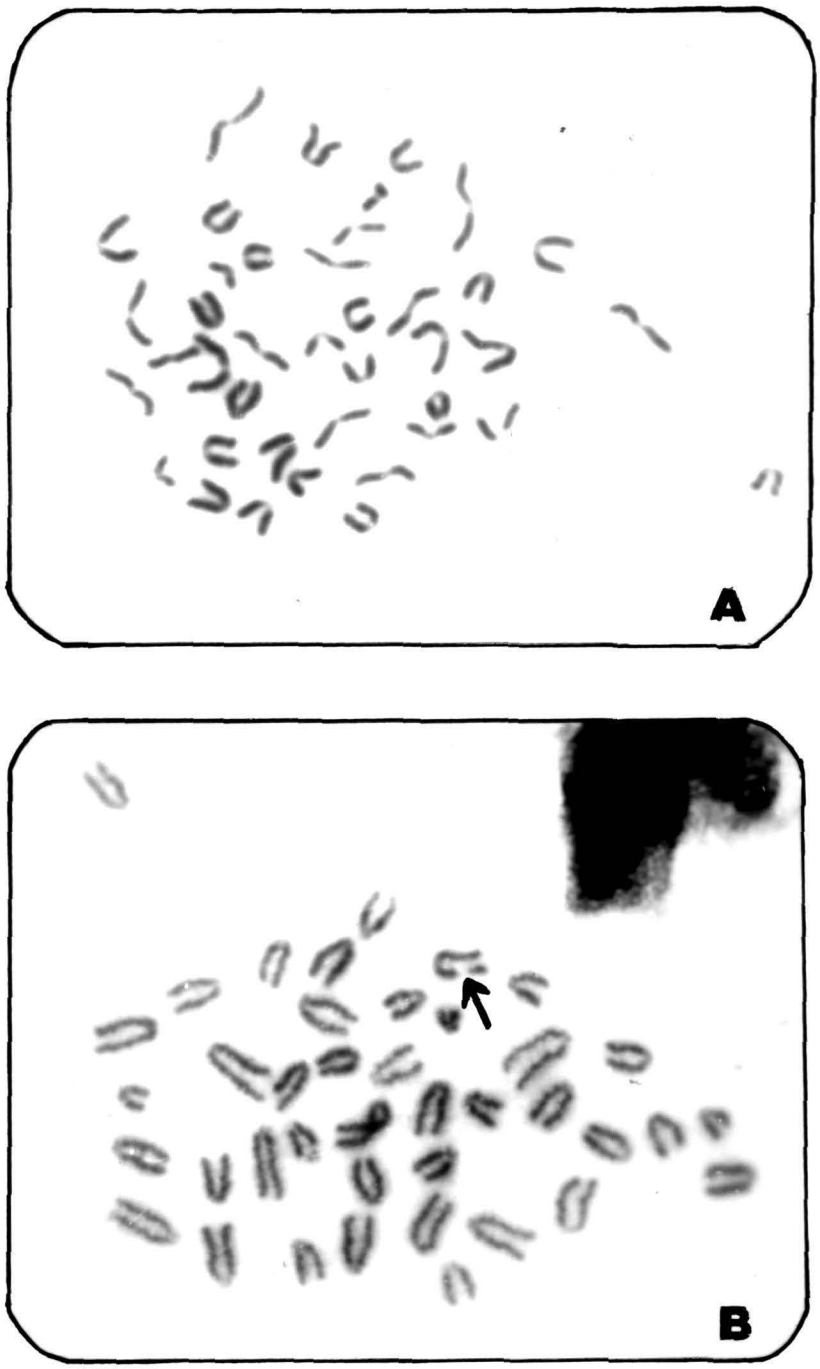


Fig 1.1: Microphotographs showing metaphase plate of mouse bone marrow cells.
A. Normal metaphase plate
B. An aberrant metaphase plate showing chromatid break (→) induced by AEBN / AAEBN.

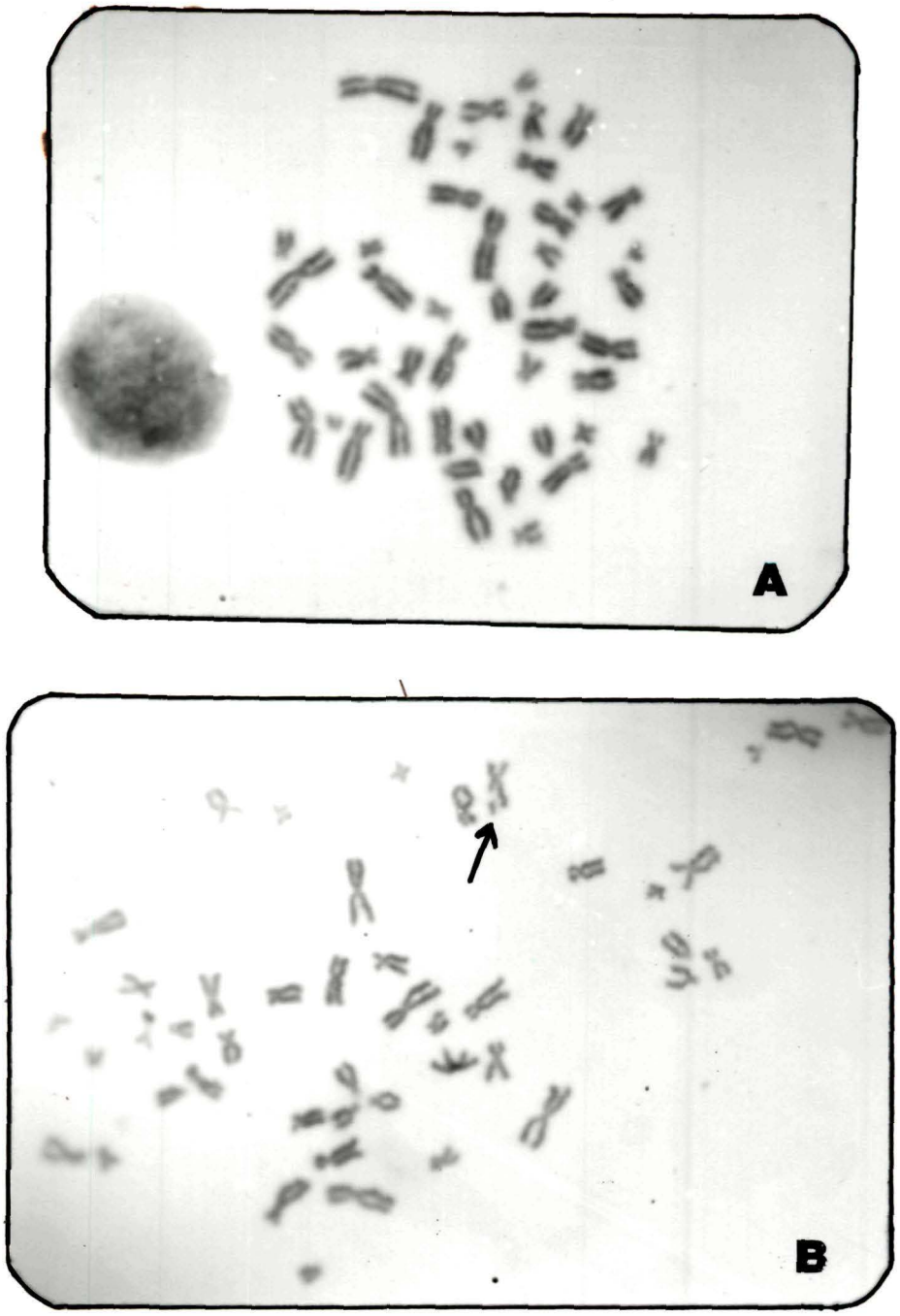


Fig 1.2: Microphotographs showing metaphase plate in Human peripheral blood lymphocytes.

A. Normal metaphase plate.

B. An aberrant metaphase plate showing chromatid break (→) induced by AEBN / AAEBN

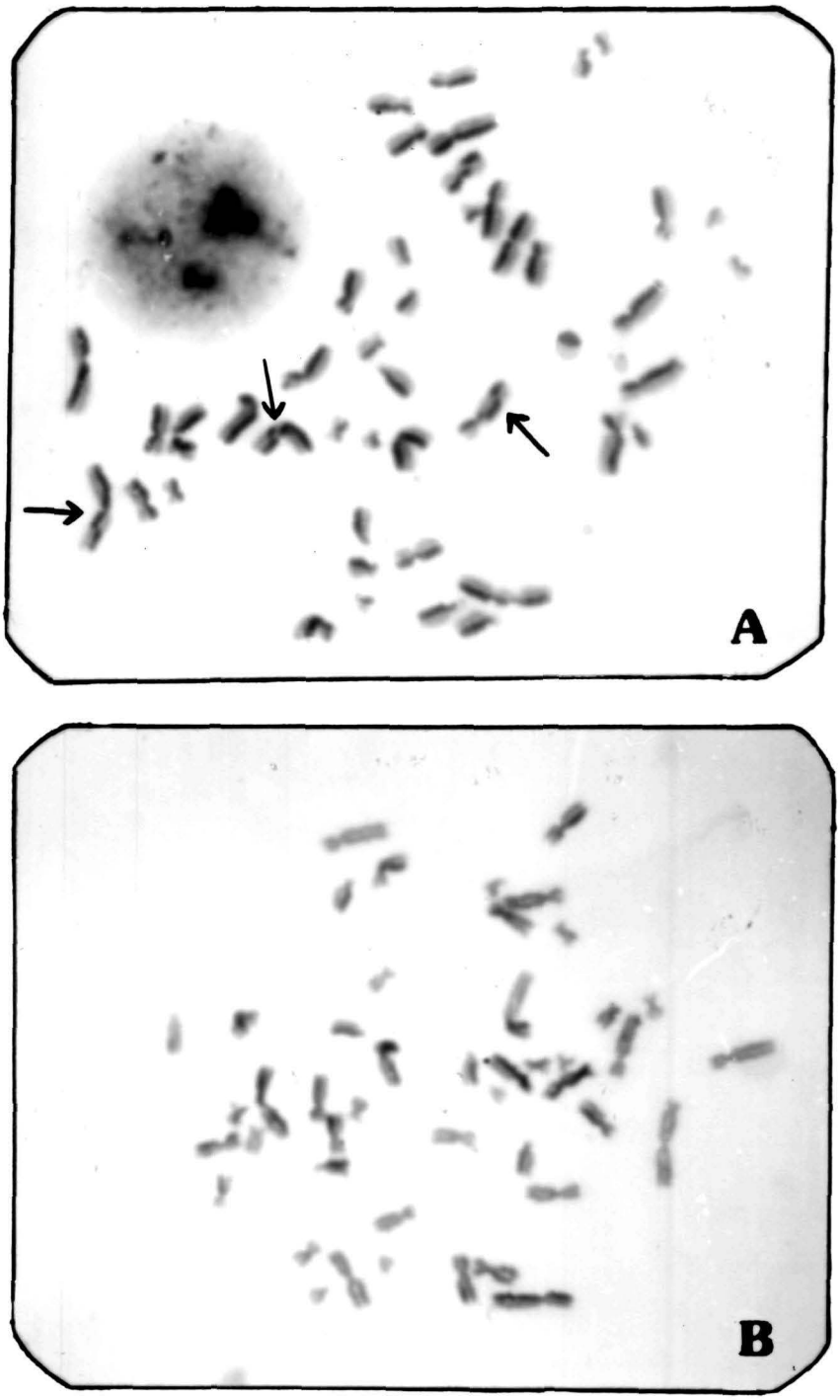


Fig 1.3: Microphotographs showing Hoechst-sunlight-Giemsa staining pattern of chromosomes in human peripheral blood lymphocytes grown in the presence of BudR

A. Second division cycle showing sister-chromatid exchanges (→).
B. Third division cycle.

Table 1.1 Concentration of total GSH in AEBN (1mg / mouse / day) and BSO (50 mg kg^{-1} b.wt.) treated and untreated mouse BMCs in vivo.

Sample #	*Treatment Days*	*Experimental Condition*	*No. of cells ($x10^{-6}$)*	*GSH ($\mu mol\ 10^{-6}$ cells)*	*Mean ± SEM (Reduction %)*
1	00	Untreated	1.95	2.00	
2			1.61	3.20	
3			0.62	3.22	
4			0.76	2.14	
5			2.12	3.15	
6			1.86	4.00	
7			1.90	3.40	
8			1.50	2.50	**2.95 ± 0.24**
1	01	BSO	1.37	1.28	
2			1.35	1.20	
3			1.10	1.82	
4			0.79	1.65	
5			0.87	1.49	**1.49[a] ± 0.11 (-49%)**
1	05	BSO	2.10	1.01	
2			2.10	1.13	
3			1.18	1.11	
4			1.72	1.13	
5			2.97	1.09	**1.09[b] ± 0.22 (-63%)**
1	01	AEBN	1.18	2.82	
2			0.83	2.01	
3			1.31	2.54	
4			0.77	2.16	
5			1.60	1.95	
6			1.35	2.04	**2.25 ± 0.14 (-24%)**
1	05	AEBN	1.26	1.33	
2			1.70	1.05	
3			1.17	1.43	
4			1.38	1.27	
5			1.80	1.11	
6			0.93	2.02	**1.37[a] ± 0.14 (-54%)**

[a] $p<0.05$, [b] $p<0.01$: student's t-test compared to untreated controls.

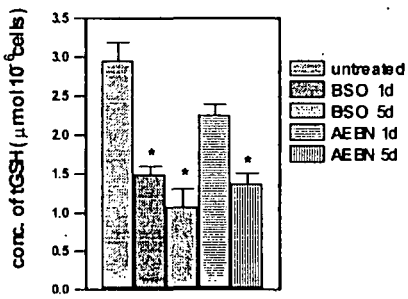


Fig 1.4 Level of tGSH in AEBN and BSO treated BMCs. Each point and bar represents a mean±SEM value. *p<0.05 as compared with control group.

Table 1.2 Induction of CAs in mouse BMCs after treating with AEBN and AAEBN (1mg / mouse / day) with or without BSO (50 mg kg^{-1} b.wt.).

Experimental Condition	*Treatment days.*	*TM*	*Abt.M (%)*	*Mean ± SEM*	*Aberration (%)* Chd.bk.	$\bar{X}$ ± SEM	Del.	$\bar{X}$ ± SEM
untreated	-	251	03		03		00	
		178	02		01		00	
		182	02		01		00	
		108	01		01		00	
		101	02		01		01	
		102	02	**02 ± 0.3**	01	**01 ± 0.3**	01	**0.33 ± 0.2**
BSO	10h	134	09		09		00	
		103	06		06		00	
		201	03		03		00	
		266	04	**06 ± 1.4**	04	**06 ± 1.4**	00	**00**
AAEBN	05	116	06		06		00	
		125	06		06		00	
		113	05		05		00	
		106	06	**06±0.3**[b]	07	**06 ± 0.5**	00	**00**
AEBN	05	096	06		06		00	
		125	09		07		02	
		110	06		06		00	
		126	10		10		03	
		120	09		09		01	
		130	11		09		03	
		148	09	**09± 0.7**[c]	09	**08 ± 0.6**	01	**01 ± 0.5**
BSO+AEBN	05	136	13		11		01	
		155	16		15		02	
		121	12		09		04	
		147	10	**13 ±1.3**[a]	09	**11 ± 1.4**	02	**02 ± 0.6**
AEBN	30	113	11		11		01	
		142	11		11		00	
		134	10		10		01	
		167	13		14		01	
		094	12		14		01	
		124	13	**12± 0.5**[c]	14	**12 ± 0.8**	01	**01 ± 0.2**
BSO+AEBN	30	110	14		14		02	
		112	15		14		03	
		112	13		13		02	
		096	13		13		02	
		108	19		19		01	
		142	13		15		01	
		164	14	**14± 0.8**	14	**15 ± 0.8**	01	**02 ± 0.3**
AAEBN	30	108	15		15		00	
		154	13		14		00	
		088	14		15		00	
		117	14	**14± 0.4**[c]	15	**15 ± 0.3**	01	**0.3 ± 0.3**
BSO+AAEBN	30	123	13		15		00	
		094	15		15		00	
		133	15		15		01	
		127	14	**14 ± 0.5**	16	**15 ± 0.3**	00	**0.3 ± 0.3**

TM- Total metaphases, Abt.M- Aberrant metaphases

[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$; 2x2 contingency χ^2-test compared to control

Table 1.3 Induction of delay in cell cycle in mouse BMCs by AEBN and AAEBN (1mg/mouse/day) with and without BSO (50 mg kg^{-1} b.wt.).

Experimental condition	*Treatment days*	*Mitotic Index* TM /Total cells	(%)	*Mean ± SEM*	*M.I (%) Range*
Untreated	-	56/1055	5.31		
		67/1476	4.54		
		49/1253	3.91		
		62/1245	4.98		
		43/0929	4.63		
		58/1133	5.12	**4.75 ± 0.2**	**3.91 - 5.31**
BSO	10h	37/1235	3.00		
		33/1375	2.40		
		20/1083	1.85		
		38/1652	2.30	**2.39 ± 0.4**	**1.85 - 3.00**
AAEBN	05	62/1623	3.82		
		37/1225	3.02		
		57/1454	3.92		
		54/1428	3.78	**3.64 ± 0.2**	**3.02 - 3.92**
AEBN	05	55/1167	4.71		
		47/1260	3.73		
		63/1491	4.23		
		44/1288	3.42		
		93/2841	3.27		
		52/1152	4.51		
		113/2480	4.56	**4.06 ± 0.2**	**3.27- 4.71**
BSO+AEBN	05	50/1013	4.93		
		70/1735	4.03		
		47/1635	2.88		
		92/1886	4.86	**4.18 ± 0.5**	**2.88- 4.93**
AEBN	30	42/1453	2.89		
		39/1912	2.04		
		51/3054	1.67		
		32/2222	1.44		
		22/2018	1.09		
		28/2545	1.10	**1.71[a] ±0.3**	**1.09- 2.89**
BSO+AEBN	30	15/1159	1.29		
		17/1532	1.11		
		21/1174	1.79		
		25/2336	1.07		
		22/1852	1.18		
		33/1375	2.40		
		22/1134	1.94	**1.54 ± 0.2**	**1.07- 2.40**
AAEBN	30	14/1108	1.26		
		20/1057	1.89		
		13/1884	0.69		
		46/1893	2.43	**1.57[a] ±0.4**	**0.69- 2.43**
BSO+AAEBN	30	12/1412	0.85		
		11/1375	0.80		
		23/1643	1.40		
		17/1932	0.88	**0.98 ± 0.1**	**0.80- 1.40**

TM- Total metaphases, M.I - Mitotic Index. [a] $p<0.01$; student's t-test compared to untreated controls.

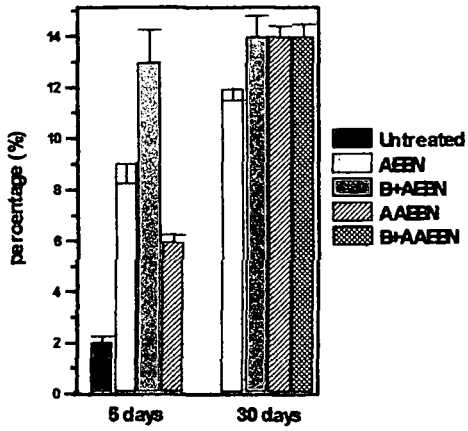


Fig: 1.5 Effect of BNE with or without BSO on the frequency of aberrant metaphases in mouse BMCs. Each point and bar represent a mean±SEM value.

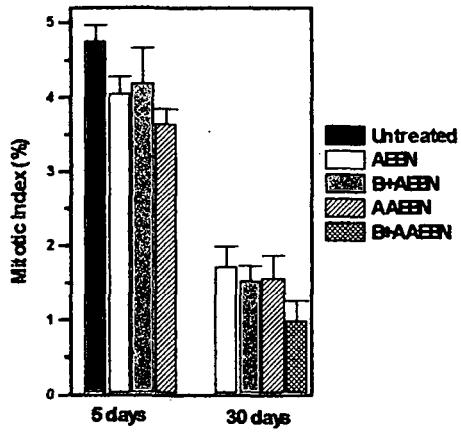


Fig: 1.6 Effect of BNE with or without BSO on cell kinetics in Mouse BMCs. Each point and bar represent a mean±SEM value.

Table 1.4 Concentration of total GSH in HPBLs with or without BSO (5mM) or RBNE (250 μg ml^{-1})

Donor #	*Experimental Condition*	*Total GSH μmol 10^{-6} cells*	*Mean ± SEM (Increment %)*
1	Untreated	6.41	
2		4.45	
3		5.19	
4		5.04	**5.27 ± 0.41**
1	BSO	0.85	
2		0.78	
3		0.98	
4		0.79	
5		0.92	**0.86[b] ± 0.04 (-84%)**
1	AEBN	7.18	
2		9.47	
3		9.56	
4		8.41	**8.66[a] ± 0.56 (64%)**
1	AAEBN	7.04	
2		8.32	
3		8.11	
4		8.19	**7.92[a] ± 0.29 (50%)**

[a]$p<0.05$, [b]$p<0.001$ student's t-test compared to the control value

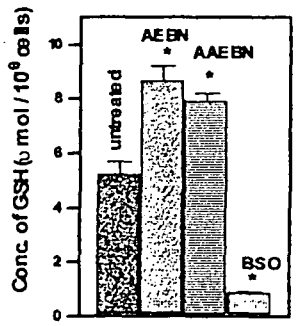


Fig 1.7 Level of tGSH in BSO and AEBN / AAEBN treated HPBLs. Each point and bar represent a mean ± SEM value. *p<0.05 compared to control.

Table 1.5 Induction of CAs and delay in cell kinetics by AEBN (μg ml^{-1}) in HPBLs in vitro (72h)

Donor #	Experimental Condition	Abt.M(%) / TM_1	Chd.bk (%)	TM_2	M1 %	M2 %	AGT (H)	Mitotic Index TM_3/Total cells	Mitotic Index (%)
1	Untreated	02/108	02	132	54	35	45.0	36/0968	3.72
	AEBN(100)	09/056	11	146	52	42	46.8	39/1340	2.91
	BSO+AEBN	12/060	15	133	57	35	48.0	24/1076	2.23
2	Untreated	01/096	01	244	45	39	41.9	16/1066	1.50
	AEBN(100)	08/145[a]	10	293	55[a]	29	44.7	33/1120[a]	2.95
	BSO+AEBN	09/211	09	409	57	32	46.8	64/1675	3.82
	AEBN(250)	13/135[b]	16	191	74[c]	17	53.7	25/1662	1.50
	BSO+AEBN	13/127	13	166	83	15	60.7	67/1994[c]	3.36
3	Untreated	02/112	02	257	47	20	38.7	45/1278	3.52
	AEBN(100)	07/174	07	334	62[c]	42	48.8	34/1762[b]	1.93
	BSO+AEBN	09/313	09	419	78[c]	15	56.3	24/1188	2.02
	AEBN(250)	14/059[b]	15	133	56	38	60.7	38/1919[a]	1.98
4	Untreated	02/096	02	194	56	24	43.9	49/2103	2.33
	AEBN(100)	08/059	08	127	58	31	48.6	21/1184	1.77
	AEBN(250)	11/100[a]	12	158	65[d]	22	49.0	33/1499	2.20
	BSO+AEBN	13/164	13	250	69	20	50.6	41/1855	2.21
	BSO(5mM)	02/091	02	198	48	33	42.1	53/1295	4.09
5	Untreated	02/090	02	241	45	21	37.9	50/1322	3.78
	AEBN(250)	10/126[a]	10	234	70[c]	19	51.1	28/1370[b]	2.00
	BSO+AEBN	11/111	13	148	75	22	56.3	26/1195	2.18
	BSO(5mM)	02/117	02	253	48	31	41.7	32/1245	2.57

Abt.M / TM_1 Aberrant metaphases / Total metaphases for aberration study; TM_2: Total cells for cell kinetics, AGT: Average generation time, TM_3: Total metaphases for mitotic index, M.I: Mitotic Index ; [a]$p<0.05$, [b]$p<0.01$,[c]$p<0.001$ 2x2 χ^2 contingency test compared to control. d: Border line of significance at $p\leq0.05$

Table 1.6 Pooled data on the induction of CAs and delay in cell kinetics by AEBN (μg ml^{-1}) in HPBLs (72h)

Donor #	Experimental Condition	Abt.M (%) ±SEM	TM_1	Chd.bk (%) ±SEM	TM_2	M1 % ±SEM	AGT(H) ±SEM	Mitotic Index TM_3 / Total cells	Mitotic Index (%) ±SEM
05	Untreated	02 ± 0.2	502	02 ± 0.2	1068	49 ± 2.3	41.5 ± 1.4	196/6737	2.97 ± 0.5
04	AEBN (100)	08 ± 0.4[a]	434	09 ± 0.9	0900	57 ± 2.1[a]	47.2 ± 1.0[#]	127/5406	2.39 ± 0.3
03	BSO + AEBN	10 ± 1.0	584	11 ± 2.0	0961	64 ± 7.0[a]	50.4 ± 3.0	115/3939	2.69 ± 0.6
04	AEBN (250)	12 ± 0.9[a]	420	13 ± 1.4	0716	66 ± 3.9[a]	50.3 ± 1.3[$]	124/6450[a]	1.92 ± 0.2
03	BSO + AEBN	12 ± 0.7	402	13 ± 00	0564	76 ± 4.1[a]	53.6 ± 3.0[@]	134/5044	2.58 ± 0.4
02	BSO (5mM)	02 ± 00	208	02 ± 00	0451	48 ± 00	41.9 ± 0.2	085/2540	3.33 ± 0.5

[a]$p<0.001$ 2x2 χ^2 contingency test compared to control [@]$p<0.05$,[#]$p<0.01$,[$]$p<0.001$ student's t test compared to controls

Table 1.7 Induction of CAs and delay in cell kinetics by AAEBN (μg ml^{-1}) in HPBLs (72h)

Donor #	*Experimental Condition*	*Abt.M(%)/ TM_1*	*Chd.bk (%)*	*TM_2*	*M1 %*	*M2 %*	*AGT (H)*	*Mitotic Index TM_3/Total cells*	*(%)*
1	Untreated	02/112	02	257	47	20	38.7	45/1278	3.52
	AAEBN(100)	10/210[a]	10	384	63[c]	28	51.8	53/2236[d]	2.37
	BSO+AAEBN	10/191	10	275	74[b]	17	53.3	41/2029	2.02
2	Untreated	01/176	01	243	32	30	34.9	77/1503	5.12
	AAEBN(100)	12/157[c]	16	300	22[a]	39	33.1	49/1493[a]	3.28
	BSO+AAEBN	13/063	14	184	39	40	39.4	30/1023	2.93
3	Untreated	01/100	01	196	52	35	44.8	42/1102	3.81
	AAEBN(100)	08/132[a]	08	124	52	25	41.9	28/1761[c]	1.59
	BSO+AAEBN	08/077	09	110	74[c]	23	55.0	29/1213	2.39
	AAEBN(250)	09/055[a]	09	187	43	36	40.2	45/1590	2.83
	BSO+AAEBN	12/094	13	182	54[a]	29	44.2	82/1760[b]	4.66
4	Untreated	01/118	01	236	52	39	45.6	59/1680	3.51
	AAEBN(100)	07/097[a]	07	198	53	32	44.1	48/1230	3.90
	BSO+AAEBN	08/080	08	177	57	28	45.4	45/1380	3.26
	AAEBN(250)	09/093[a]	10	213	47	28	40.6	30/0946	3.17
	BSO+AAEBN	10/103	10	217	55	35	46.5	32/1142	2.80
5	Untreated	02/090	02	241	45	21	37.9	50/1322	3.78
	AAEBN(250)	10/070	10	243	33[b]	28	34.3	58/1424	4.07
	BSO+AAEBN	13/079	13	277	31	29	34.5	53/1471	3.60
6	Untreated	03/112	03	242	49	29	41.6	67/1704	3.93
	AAEBN(250)	10/099	10	222	54	35	45.6	25/1086[a]	2.30
	BSO+AAEBN	11/071	11	179	44[d]	31	39.8	19/1032	1.84
	BSO(5mM)	02/117	02	253	48	31	41.6	32/1245	2.57
7	Untreated	02/096	02	194	56	24	43.9	49/2103	2.33
	AAEBN(250)	10/114[a]	10	244	49	33	40.9	24/1175	2.04
	BSO+AAEBN	10/117	10	235	54	25	43.4	28/1064	2.63
	BSO(5mM)	02/091	02	198	48	33	42.1	53/1295	4.09

Abt.M/TM_1: Aberrant metaphases / Total metaphases in aberration study; TM_2: Total cells for cell kinetics, AGT: Average generation time, TM_3: Total metaphases for mitotic index; M.I: Mitotic Index; [a]$p<0.05$, [b]$p<0.01$,[c]$p<0.001$ 2x2 χ^2 contingency test compared to control;d: border line of significance at $p<0.05$ level

Table 1.8 Pooled data on the induction of CAs and delay in cell kinetics by AAEBN (μg ml^{-1}) in HPBLs (72h)

Donor #	*Experimental Condition*	*Abt.M (%) ±SEM/*	*TM_1*	*Chd.bk (%) ±SEM*	*TM_2*	*M1 % ±SEM*	*AGT (H) ±SEM*	*Mitotic Index TM_3 / Total cells*	*(%) ±SEM*
07	Untreated	02 ± 0.4	804	02 ± 0.4	1609	48 ± 2.9	41.1 ± 1.5	389 / 10692	3.72 ± 0.3
04	AAEBN (100)	09 ± 1.1[a]	596	10 ± 2.3	1006	48 ± 8.9	42.8 ± 3.8	178 / 6720[a]	2.79 ± 0.5
04	BSO + AAEBN	10 ± 1.2	411	10 ± 1.3	0746	61 ± 8.4[a]	48.3 ± 3.6	145 / 5645	2.65 ± 0.3
05	AAEBN (250)	10 ± 0.2[a]	431	10 ± 0.4	1109	45 ± 3.5	40.3 ± 1.8	182 / 6221[a]	2.88 ± 0.4
05	BSO + AAEBN	11 ± 0.6	464	11 ± 0.6	1090	48 ± 4.6	41.7 ± 2.1	214 / 6473	3.11 ± 0.5
02	BSO (5mM)	02 ± 00	208	02 ± 00	0451	48 ± 00	41.9 ± 0.2	85 / 2540	3.33 ± 0.5

[a] $p<0.001$ 2x2 χ^2 contingency test compared to control.

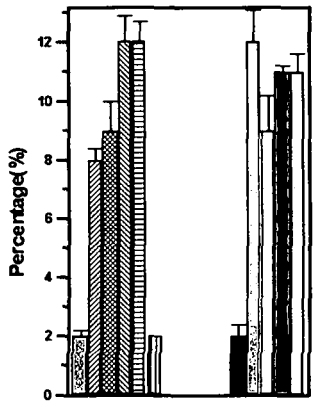
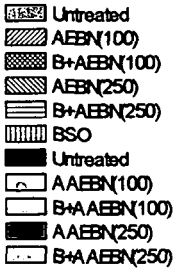


Fig:1.8 Effect of AEBN / AAEBN with or without BSO on the freuency of aberrant metaphases in HPBLs. Each point and bar represents a mean±value.



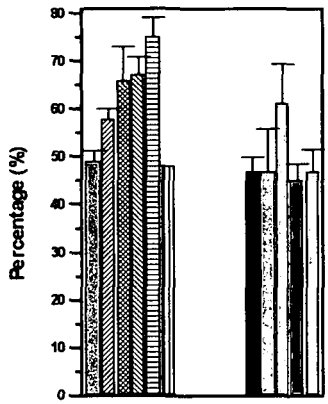


Fig:1.9 Effect of AEBN / AAEBN with or without BSO on the frequency of percentage of M1 cells in HPBLs. Each point and bar represents the mean±SEM value.

Table 1.9 Induction of SCEs by AEBN (μg ml^{-1}) in HPBLs in vitro (72h)

Donor #	Experimental Condition	Cells Scored	SCE / M	SCE /M (%) 0-3	4-6	7-10	11-15	>15	H
1	Untreated	29	5.75	21	55	14	10	00	1.68^{S}
	AEBN(100)	42	7.33	07	38	38	17	00	0.58
	BSO+AEBN	28	8.58	04	29	39	29	00	1.18
2	Untreated	36	5.76	14	50	36	00	00	0.85
	AEBN(100)	55	6.25	07	53	40	00	00	0.54
	BSO+AEBN	75	6.91	07	45	40	08	00	1.01
	AEBN(250)	22	8.32[a]	00	05	91	05	00	0.19
	BSO+AEBN	20	9.65	00	20	40	30	10	1.69^{S}
3	Untreated	32	5.54	16	59	25	00	00	1.06
	AEBN(100)	42	6.36	10	45	43	02	00	0.90
	BSO+AEBN	35	7.83	03	29	51	17	00	1.05
	AEBN(250)	31	10.65[a]	00	03	48	48	00	0.53
4	Untreated	29	6.48	07	52	31	10	00	0.98
	AEBN(100)	28	7.71	04	43	32	21	00	1.41
	AEBN(250)	34	10.41[a]	00	24	26	41	09	1.54^{S}
	BSO+AEBN	32	11.22	00	09	41	31	19	1.38
	BSO(5mM)	54	5.57	22	43	30	06	00	1.05
5	Untreated	32	5.16	16	66	16	03	00	0.73
	AEBN(250)	20	6.00	20	45	35	00	00	0.88
	BSO+AEBN	33	6.94	03	48	39	09	00	1.01
	BSO(5mM)	39	5.97	21	46	28	05	00	0.59

H-Dispersion coefficient = variance/mean, $ significantly different at α= 0.05 from Poisson distribution; [a] $p<0.01$, Student's t-test compared to controls.

Table 1.10. Pooled data on the induction of SCEs by AEBN (μg ml^{-1}) in HPBLs in vitro (72h)

Donor #	Experimental Condition	Cells Scored	SCE / M ±SEM	SCE /M (%) 0-3	4-6	7-10	11-15	>15	H
05	Untreated	158	5.74 ± 0.2	15	56	24	05	00	1.06
04	AEBN(100)	167	6.91 ± 0.4	07	45	38	10	00	0.86
03	BSO+AEBN	138	7.77 ± 0.5	05	34	43	18	00	1.08
04	AEBN(250)	107	8.85 ± 1.1[a]	05	19	50	24	02	0.79
03	BSO+AEBN	085	9.27 ± 1.3	01	26	40	23	10	1.36
02	BSO(5mM)	093	5.77 ± 0.1	22	44	29	05	00	0.82

[a] $p<0.05$ student's t test compared to controls

Table 1.11 Induction of SCEs by AAEBN (μg ml^{-1}) in HPBLs (72h)

Sample #	Experimental Condition	Cells Scored	SCE/ M	SCE /M(%) 0-3	4-6	7-10	11-15	>15	H
1	Untreated	32	5.54	16	59	25	00	00	1.06
	AAEBN(100)	54	8.50^{c}	02	24	50	30	04	1.07
	BSO+AAEBN	30	8.53	00	13	63	23	00	0.65
2	Untreated	47	7.51	09	36	36	17	02	1.73$^{\$}$
	AAEBN(100)	71	9.97^{b}	01	11	44	41	03	0.94
	BSO+AAEBN	39	10.57	00	05	59	28	08	1.12
3	Untreated	39	5.94	13	49	38	00	00	0.66
	AAEBN(100)	22	7.77^{a}	05	23	59	14	00	0.66
	BSO+AAEBN	13	8.15	00	38	38	23	00	1.22
	AAEBN(250)	36	7.72^{a}	06	39	33	22	00	1.40
	BSO+AAEBN	27	9.42	00	19	48	33	00	1.13
4	Untreated	42	5.48	21	50	29	00	00	0.71
	AAEBN(100)	38	6.68	11	39	42	08	00	1.06
	BSO+AAEBN	37	8.18	11	24	43	19	03	1.69$^{\$}$
	AAEBN(250)	34	9.73^{c}	00	15	53	29	03	1.12
	BSO+AAEBN	32	10.06	06	03	53	31	06	1.01
5	Untreated	32	5.16	16	66	16	03	00	0.73
	AAEBN(250)	45	6.29	07	49	38	07	00	1.02
	BSO+AAEBN	37	7.54	05	27	51	16	00	0.87
6	Untreated	52	5.42	25	48	21	06	00	1.19
	AAEBN(250)	44	8.11^{b}	07	25	48	16	05	1.34
	BSO+AAEBN	24	10.57^{a}	00	13	42	33	13	1.12
	BSO(5mM)	39	5.97	21	46	28	05	00	0.59
7	Untreated	29	6.48	07	52	31	10	00	0.98
	AAEBN(250)	27	9.70^{b}	04	30	26	26	15	1.95$^{\$}$
	BSO+AAEBN	39	9.92	03	23	26	38	10	1.63$^{\$}$
	BSO(5mM)	54	5.57	22	43	30	06	00	1.05

H Dispersion coefficient= variance/mean, $ significantly different at α= 0.05 from Poisson distribution ; $^{a}p<0.05$,$^{b}p<0.01$,$^{c}p<0.001$ student's t test compared to controls.

Table 1.12 Pooled data on the induction of SCEs by AAEBN (μg ml^{-1}) in HPBLs in vitro (72h)

Donor #	Experimental Condition	Cells Scored	SCE/M ±SEM	SCE /M(%) 0-3	4-6	7-10	11-15	>15	H
07	Untreated	273	5.93 ± 0.3	15	51	28	05	00	1.01
04	AAEBN(100)	185	8.23^{a} ± 0.7	05	24	49	23	02	0.96
04	BSO+AAEBN	119	8.86 ± 0.6	03	20	51	23	03	1.17
05	AAEBN(250)	186	8.31^{a} ± 0.6	05	32	40	20	05	1.37
05	BSO+AAEBN	159	9.50 ± 0.5	03	17	44	30	06	1.15
02	BSO(5mM)	093	5.77 ± 0.1	22	44	29	05	00	0.82

^{a} $p<0.05$ student's t test compared to controls

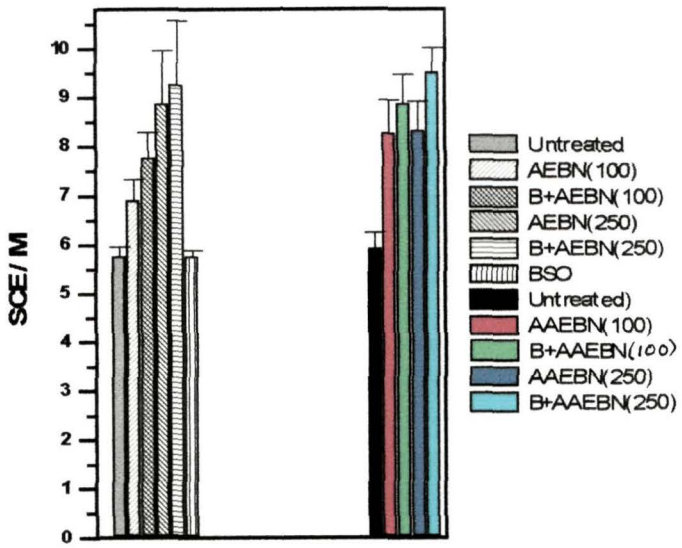


Fig:1.10 Effect of AEBN /AAEBN with or without BSO on the frequency of SCEs in HPBLs. Each point and bar represents the mean ±SEM value.

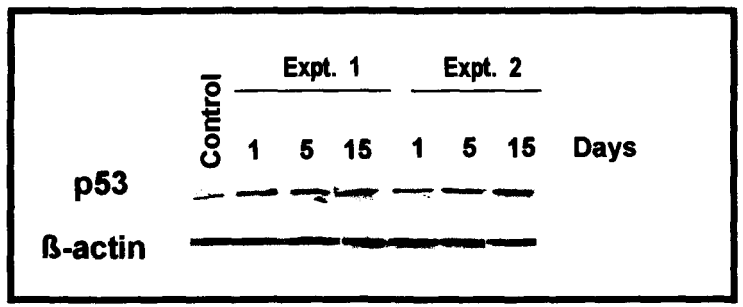


Fig. 1.11 The level of p53 protein in mouse bone marrow cells after RBNE exposure

Discussion

In order to understand betel-nut induced genotoxicity we studied the effect of unprocessed betel-nut extract (RBNE) on cell kinetics, CA and SCE induction in both in vivo and in vitro systems with respect to endogenous GSH status. All these cytogenetical parameters are considered to be sensitive indicators of DNA damage which increases the risk of cancer and genetic ill health (Chagnati et al 1974, Buckton et al 1978). The present data indicate that RBNE induced significant delay in cell kinetics, SCE and CAs and the depletion of endogenous GSH by BSO-treatment enhanced the delay in cell kinetics and SCEs but not the frequency of CAs. It has been shown earlier that BNE can induce DNA strand breaks, SCEs and micronuclei in various kinds of cells (IARC 1985, Sundqvist et al 1989, Jeng et al 1999). BNE also can induce early malignant changes in hamster check pouch (Suri et al 1971, Ranadive et al 1976). There are also reports regarding induction of CA and SCEs in PBLs of BN-chewers (Dave et al 1992). In this study RBNE induced mostly chromatid breaks and failed to induce any exchanges. Similar observation was made with arecoline, a prominent alkaloid of betel-nut, in mouse BMCs (Deb and Chatterjee 1998). This implies that DNA lesions which are induced by RBNE may not be appearing at the same time or not in close proximity so that they failed to associate to form exchanges (Kihlman 1977). However, one can not rule out the possibility that BNE-induced DNA lesions are of different nature which are unable to form exchange aberrations since BNE elicits a reduction of the high mobility group of proteins and their poly-ADP-ribosylation may influence overall chromosomal organization and structure (Sharan 1996).

In this study both AEBN and AAEBN induced similar level of CA in mouse BMC and CA and SCEs in human PBLs. There are reports which suggest that higher acidic pH favors accumulation of nitrite and consequently facilitate carcinogenic transformation (IARC 1985, Ohshima et al 1989). However, it seems that in this

study higher acidic pH with AAEBN was unable to induce more CA in contrast to aqueous extract till 30 days of continuous treatment.

A novel aspect of the present study is the analysis of the influence of BSO on the BNE-induced CAs, SCEs and delay in cell kinetics. The rationale for BSO-treatment is based on the premise that GSH serves as a major endogenous cellular defence against various toxic effects of xenobiotics (Kosower and Kosower 1978, Williamson et al 1982) and GSH-depletion itself may lead to significant sensitization. Treatment with BSO produces a rapid decrease in the GSH levels of the various tissues (Griffith and Miester 1979) and in the present study 49% depletion of GSH with respect to control was obtained. It has been demonstrated that following a single dose of 556 mg kg^{-1} BSO for 8-12h 83% depletion was achieved for the mouse BMCs (Lee et al 1987). Therefore, the incubation period of BSO-treatment in mouse BMCs was kept for 10h in the present study. In earlier study we used 200 mg kg^{-1} as a single dose and 54% depletion of the initial level was achieved (Deb and Chatterjee 1998, Chattopadhyay et al 1999). In this study since we used BSO more than once, low concentration of BSO was used to avoid any possible toxic effect of BSO on cells since such toxic effect was reported earlier (Dethlefsen et al 1988, Revesz et al 1994). In case of in vitro study the freshly drawn blood was incubated with BSO for 5h since in cultured cells more than 75% depletion was achieved within 4-5h duration by 500μm to 10mM of BSO (Shrieve et al 1985, Edgren and Revesz 1987). In this study the concentration of BSO used was 5mM and 84% depletion was obtained after 3h exposure with respect to untreated control.

It appears that BSO-mediated GSH-depletion increases the number of DNA strand break induction by BNE thereby enhancing the frequency of SCEs significantly and CA marginally. In fact in our earlier studies intraperitoneal injection of ARC (1 mg / mouse) to BSO-treated mouse significantly increased the frequency of CA and SCEs in BMC. In contrast to the earlier studies the inability to increase the frequency of CA by RBNE in BSO-treated mouse in the

present study could be due to low exposure of mouse to ARC in 1 mg RBNE per day. In addition to this the route of treatment is also different.

The present data show that RBNE induced SCEs significantly in a dose dependent manner and the prior treatment with BSO increased the frequency of BNE-induced SCEs significantly. It is clear from the data that BNE could induce DNA strand breaks which is further increased in presence of BSO. In BNE-treated sample the frequency of cells having 7 or more SCEs were higher than control and when BSO along with AEBN or AAEBN was treated the cells having 11 or more SCEs per cell were increased substantially in most of the experiments. The dispersion analysis indicated that the distribution of SCEs per cell after RBNE treatment combined with BSO showed Poisson distribution which is an indication that the SCE-induction in those samples was due to DNA damage since DNA damaging agent that induces SCEs fit well with the Poisson distribution (Rainaldi and Mariani 1982). It was suggested that there are at least two mechanisms of SCE induction. One is by damaging DNA (MacRae et al 1979) and another by inhibiting DNA synthesis (Ishii and Bender 1982). It seems that both the mechanisms of SCE induction could take place after BNE treatments since inhibiting DNA synthesis could lead to block cell proliferation which was also observed in RBNE-treated cells.

In this study, RBNE induced significant delay in cell cycle in both systems. It has been reported that BNE can decrease the clonal cell growth of buccal epithelial cells (Sundqvist et al 1989), gingival keratinocytes (Jeng et al 1999) and fibroblast cells (VanWyk et al 1994). Earlier arecoline showed the ability of induction of delay in cell kinetics in mouse BMC (Deb and Chatterjee 1998) and similar observation was made by others in human gingival fibroblasts (Jeng et al 1996) and also in skin fibroblasts (Van Wyk et al 1995). The present data demonstrated that the depletion of endogenous GSH enhanced the induction of delay in cell kinetics caused by RBNE. It is known that ARC, a component of BNE, by losing only one of its methyl group (Boyland and Nery 1969), may bind

with nucleic acid and protein (Nery 1971) and by doing so it could induce delay in cell kinetics. However, present data show that the RBNE-treatment alone deplete endogenous GSH in mouse BMCs and this could also be responsible for the delay since cellular GSH are important for cell proliferation (Jeng et al 1996). Depletion of cellular GSH by the treatment of ARC and RBNE has been reported earlier (Sundqvist et al 1989, Zain et al 1997, Jeng et al 1996) and it render the cells susceptible to potential further attack by metabolic BN byproducts. Consistent with these findings it has been shown that the extracellular addition of GSH, cysteine and N-acetyl-L-cysteine (NAC) could prevent ARC cytotoxicity (Jeng et al 1994, Chatterjee and Deb 1999). In contrast to mouse BMC the human PBLs showed enhanced GSH level after RBNE-treatment. Reasons behind such enhancement is not clear, however, cell type, method of exposure, BN concentration, species difference in enzymes necessary for the metabolic activation of BN ingredients could partly be responsible for the observed response.

It is worth noting that inspite of initial differences in the GSH-level in response to RBNE-treatment in both the cell system RBNE could induce delay in cell kinetics and CA. Although it is observed that in human PBLs AEBN induced significant delay in cell kinetics in most of the experiments and such induction was not their in case of AAEBN. The reason for this is not clear.

The present data indicate that RBNE could induce CA and delay in cell kinetics by inducing DNA damage and lowering endogenous GSH level in mouse BMCs. Further depletion of GSH by BSO enhanced the frequency of such damages. The extent of damage for each parameter was more when mouse was exposed continuously to BNE for 30 days than 5 days. 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 (Hall et al 1993, Zhan et al 1994), by blocking the damaged cells at the G1 checkpoint and then presumably allowing time for DNA repair (Smith et al 1995). Thus the present observed

increase in delay after 30 days exposure may be explained as higher induction of DNA damage and therefore, more time was required for repair of such damage. If this is true then longer exposure of RBNE should show higher level of p53 expression and the present result indeed showed higher p53 level after 15 days exposure than 1 and 5 days. 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 (Kastan et al 1991). Increased expression of p53 protein has also been reported in head and neck squamous cell carcinoma (Chiba et al 1996, Ahomadegbe et al 1995). Moreover, elevated levels of p53 protein have been observed not only in oral squamous cell carcinomas but also in oral dysplastic lesions, suggesting that p53 alteration is an early event in oral oncogenesis (Kaur et al 1994).

Therefore, it may be concluded that RBNE has the genotoxic ability which is further enhanced by depletion of endogenous GSH level. Habitual chewers of the North-Eastern region of India chew raw betel-nut, lime and betel leaf 10-12 times or more a day for 10-20 min each time and hence the duration of exposure in the mouths of humans is many times longer than that expected in our murine system. Such continuous exposure may attack the already present DNA-damaged cells or the cells which has just repaired and thereby activate the repair machinery for another round. This repeated repair may lead to error-prone repair and ultimately leading to altered gene expression. The increased production of COX-2 protein by gingival keratinocytes was observed following exposure to BNE (Jeng et al 2000) and overproduction of COX-2 mRNA in head and neck tumour tissues has been reported (Chan et al 1999). During the process of continuous exposure p53 gene could be a target gene and such tumour suppressor gene mutation are the most common genetic abnormalities in human cancers, especially in the development of head and neck cancer (Harris 1996, Liloglou et al 1997). Therefore, the present result indicate that the endogenous GSH-level and p53 expression can provide measure of the exposure, toxic effect

and individual susceptibility to environmental chemical compound such as raw betel-nut and may be very useful to assess and control the risk of long-term outcomes associated with exposure to xenobiotics.

CHAPTER II

CELL CYCLE KINETICS, GSH STATUS AND LEVEL OF p53: BIOMARKERS OF DNA DAMAGE IN BETEL NUT CHEWERS

Literature review

The process of mutagenesis and carcinogenesis is complicated because many factors are involved eg, DNA lesions, DNA repair, cell division, clonal instability, apoptosis and level of p53-protein (Christensen et al 1999, Hill et al 1999). When chemical damage occurs as a consequence of exposure to exogenous agents, either chemical or physical, these agents are generally carcinogenic and the type of damage and mutations they induce can act as a molecular fingerprint indicating exposure to these environmental carcinogen (Greenblatt et al 1994, Multani et al 2000). There is growing concern about possible mutagenic and carcinogenic effects of genotoxic agents in human populations exposed occupationally, accidentally or by life style. The most extensively employed method to assess the genetic effects of such exposures has been the analysis of chromosomal alterations in stimulated peripheral blood lymphocytes of exposed persons. These HPBLs is a very useful system for monitoring human populations exposed to genotoxic chemicals. HPBLs are used so that the cytogenetic effects of BN at sites other than the oral cavity and esophageal lining (target tissues) can be carried out (non-target tissue). The system thus offers an advantage to test the *in vivo* mutagenic / genotoxic effects of chemicals on somatic cells.

Oral squamous cell carcinoma is the most common malignancy in India (Nair et al 1986) and it is tempting to consider a causal association with the region-specific habit of betel quid chewing, a hypothesis that is strengthened by epidemiological studies (Sanghvi 1981). Maher et al (1994) demonstrated that betel nut (BN) users, independently of tobacco use, are many fold more likely to get oral submucous fibrosis, which is a precancerous lesion of the mouth. The chemical composition and pharmacological actions of BN have been reported and reviewed by several workers (Arjungi 1976, Mazumdar et al 1982, Jeng et al 2001). There are strong indications for a causal association between BN or quid chewing habit and oral mucosal diseases such as leukoplakia, oral submucous

fibrosis and oral cancer (IARC 1985, Ko et al 1995). Mutagenicity and genotoxicity of betel alkaloids, polyphenol and tannin fractions have been reported (Jeng et al 2001, Panigrahi and Rao 1986, Azuine and Bhide 1992, Deb and Chatterjee 1998). BNE is mutagenic in Chinese hamster V79 cells (Shirname et al 1984) and can induce cancerous lesions on the hamster cheek pouch (Ranadive et al 1979) and also in stomach (Bhide et al 1979). The subcutaneous injection of BN extract leads to transplantable fibrosarcoma at the site of injection for 60% of Swiss mice involved (Ranadive et al 1976).

The increased frequency of SCEs, CAs and micronucleated cells in exfoliated cells of the buccal mucosa among BN chewers was reported by Dave et al (1991,1992). The frequency of lymphocytic SCEs was elevated in BN chewers and oral cancer patients in comparison to non-chewers controls (Adhvaryu et al 1991). This shows that BN not only affects the target site i.e the oral cavity but also the non-target tissues. Hazardous chemicals which are multi-site and multi species animal carcinogens are considered to pose a greater threat to humans than single-site and single species carcinogens (Tennant 1993). The betel quid that is consumed here consists of betel leaf, raw betel nut and lime. The inclusion of chewing tobacco, spices or perfumes is generally 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. The incidence of oral cancer for individuals who smoke, drink alcohol and chew BQ has been reported to be 123 fold higher than for abstainers (Ko et al 1995). Long term exposure to BQ may give rise to OSF that exhibits clinically-palpable fibrous bands tough, and leathery mucosal texture, a blanching of the oral sensation and limited mouth opening capability, burning sensation and pain leading to difficulties with mastication and phonation (Caniff et al 1986, Pillai et al 1992, Zain et al 1997, Meghji and Warnakulasuriya 1997). Besides the carcinogenic effects of BN specific nitrosamines, the presence of lime escalates the damaging

effects of betel quid. Due to presence of lime ($Ca(OH)_2$) as a major component in a betel quid preparation, BQ chewers saliva typically changes from an approximately neutral to an alkaline condition. Rosin (1984) noted that AN ingredients can release ROS under alkaline conditions and induce the mitotic conversion of *Saccharomyces cerevisiae*. When the pH level is greater than 9.5 reactive oxygen species are also capable of inducing nucleotide modification and the formation of 8-hydroxydeoxyguanosine (8-OH-dG), this process being mediated by the production of hydroxyl radicals (Nair et al 1987). Since the presence of 8-OH-dG can easily lead to the formation of mutated initiated cells during replication, the compound has been preferred as a biomarker for the attack of chemical carcinogens (Marnett, 2000, Kuchino et al 1987). The normal mucosal epithelial cells (target tissue) or any other non target tissue are continuously subjected to the attack of genotoxic agents present in BQ, tobacco, alcohol or nitrosamines and ROS (IARC 1985, Sen et al 1989, Sharan 1996, Hoffmann et al 1994). Antioxidants such as GSH, NAC and enzymes such as glutathione peroxidase, catalase and superoxide dismutase can form conjugates with ROS and reactive intermediates, thus degrading reactive toxic species and protecting the critical cellular macromolecules (DNA, proteins, membrane lipids) against oral toxicants (Kehrer 1993, Amstad and Cerutti 1990). Repeating and continuous exposure of oral mucosal cells to BQ ingredients, however, will lead to the impairment of cellular defense systems. Since alcohol, ROS and ingredients of BQ, tobacco and related nitrosamines have been shown to exert genotoxicity and are crucial for tumour initiation, promotion and progression, exposure to these toxicants simultaneously has been shown to markedly potentiate the oral cancer incidence.

The genotoxic potentials of BN/BQ have been revealed by *in vitro* short-term experiments on mammalian test systems. To substantiate the information gained by the *in vitro* studies and with a view of assessing the potential hazards associated with BN, we have attempted to determine its genotoxic potentials in

individuals regularly consuming BN. A very high frequency of micronucleated cells has been observed among Indians chewing betel quid, betel nut and/or tobacco (Stich et al 1982, a, b). From this North-Eastern region, a high frequency of occurrence of micronucleated cells in buccal mucosa of people who chew raw BN has been reported (Stich et al 1982, Stich et al 1983). Chromosome damaging activity has also been reported in the saliva of people who chew BN (Stich and Stich 1982). BN chewing may be a major risk factor in the etiology of oral and digestive system associated cancers, which account for over 50% of the total cancer incidence in India (Sanghvi 1981). It has been shown that urinary tract and urinary bladder are likely to be susceptible to cancer due to BN or BQ chewing (Trivedi et al 1995). All the four nitrosamines have been detected from the saliva of BN/BQ chewers (Prokopczyk et al 1987). Trace of N-(methyl-nitrosamino) propionitrile (NMPN) a powerful carcinogen in F344 rats (Wenke et al 1984) have been detected in saliva of those who chew betel quid without tobacco (Nair et al 1987) and have been found to have organ specific carcinogenic activity (Rivenson et al 1988). Stich and Anders (1989) reported the appearance of N-nitrosoguvacine and relatively large amounts of tannins in the saliva of BQ chewers and the saliva was genotoxic to CHO cells. The carcinogenicity of N-nitrosoguvacine is an open question with two contradictory reports (Rivenson et al 1988, Lijinsky and Taylor 1976). The *in vitro* experiments and endogenous formation of nitrosamines imply that the nitrosation of BN and /or tobacco alkaloids occurs in the mouth leading to increased exposure of buccal mucosa to specific nitrosamines. However, chewers who swallow the betel quid, may form even higher amounts of these nitrosamines in their stomach at pH 2.0 (Nair et al 1986). The esophageal lining is bathed with the genotoxins present in BN and so the possibility of more severe genotoxic effects due to BN increases.

As GSH is the major antioxidant and a very important detoxifying agent protecting the body from free radicals and also eliminates undesirable toxins and

pollutants. The changes in the antioxidant levels are likely to play a role in the induction of complications of the disease. These changes in the antioxidant levels were correlated with the duration of the disease and with the development of complications (Sundaram et al 1996). DeZwart et al (1999) proposed that the disturbances in the antioxidant systems might be useful indicators of the susceptibility of subjects to free radical damage. The exposure of buccal keratinocytes to BN and ARC leads to GSH depletion (Sundqvist et al 1989), with no concomitant rise in glutathione disulfide levels, suggesting that the toxicity of BN is possibly not mediated directly by ROS. From our studies we see that the exposure of mice to BN-extract for 1 and 5 days show higher level of depletion of endogenous GSH in bone marrow cells in 5 days exposed samples than 1 day. 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 defence against radiation (Held et al 1982, Chatterjee and Jacob-Raman 1986, Chattopadhayay et al 1999) and various toxic effects of xenobiotics (Shaw and Chou 1986, Dev-Giri and Chatterjee 1998, Syng-ai and Chatterjee 2002) but not against radiomimetic drugs like bleomycin (Chatterjee et al 1989, Chattopadhyay et al 1997). Therefore, the alteration in the level of endogenous GSH by BN ingredients will modulate the host susceptibility to the action of other chemical carcinogens.

Tumour suppressor gene p53 mutations are the most common genetic abnormalities found in human cancers, especially in the development of head and neck cancers (Sidransky and Hollstein1996, Liloglou et al 1997). 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 (Kaur et al 1994). 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 central role of p53 in eliminating the genomic damage so central to the successful genesis of

the cancer cell is reflected by the fact that over 70% of human cancers have defects in this gene and virtually all have defects in gene upstream or downstream of p53 function (Levine 1997). The determination of the levels of expression of p53 protein will also serve as an important biomarker which will reflect changes occurring in the system of a BN chewer related to exposure to BN. The purpose is surveillance, that is the identification of individuals or population at risk to adverse health effects so that preventive measures can be taken.

Damaged bases and chromosome breaks may give rise to mutations during cell division. To maintain the integrity of their genomes, higher eukaryotes have developed mechanisms to recognize and repair DNA damage prior to cell division. Failure in one of these mechanisms could be a key step in tumour progression. Loss or inactivation of the p53-tumour suppressor protein correlates genetic instability (Donehower et al 1992). In normal cells, signals arising from damaged DNA leads to activation of the p53 response pathway, resulting in cell-cycle arrest, DNA repair and apoptosis of certain cells (Harris 1996). Exposure of cells to genotoxic agents such as those used in the treatment of cancer, results in an induction of the levels of p53 that can mediate either a delay in cell-cycle progression or the induction of cell death via an apoptotic pathway (Ko and Prives 1996, Levine 1997). p53 immunoreactivity has been associated with aggressive clinical course in several tumors , including carcinomas of the lung, breast and prostrate (Quinlan et al 1992, Thor et al 1992, Visakorpi et al 1992). p53 overexpression detected immunohistochemically correlate with TP53 gene mutations in some , but not all cases of non-Hodgin's lymphoma (NHL) (Sander et al 1993, Jaslow et al 1994, Cesarman et al 1992). Interestingly immunohistochemical staining for PCNA by Cox and Walker (1995) has also elucidated that the number of proliferative basal epithelial cells is greater in BQ chewers with oral submucous fibrosis than in non-chewers, although the epithelial cells are normal or atrophic. Recent immunohistochemical studies have shown that over

expression of p53 indicates that a poor prognosis in a variety of malignant tumors including bladder, lung, breast and gastrointestinal cancers (Allred et al 1993, Iwaya et al 1991, Martin et al 1992, Quinlan et al 1992, Sarkis et al 1993, Thor et al 1992). Conversely, some investigators have reported that p53 accumulation does not affect the prognosis (Brambilla et al 1993, Scott et al 1991) but rather seems to be a biologic marker indicative of improved survival potential (Sauter et al 1992). Exposure to radiation leads to an increase in the levels of protein that derives from an alteration in its half-life as a result of post-translational modifications (Levine 1997, Ko and Prives 1996). Similar observations were made with exposures to bleomycin and other chemotherapeutic drugs (Lu and Lane 1993). Overexpression of p53 protein has been found in 40-70% of head and neck carcinomas and in one-half on the non-malignant epithelia adjacent to positive tumours (Warnakulasuriya and Johnson 1992, Field et al 1991, Shin et al 1994).

The North-East Indian variety of BN is raw, wet and unprocessed consumed with betel-leaf and slaked lime. Stich et al (1985) demonstrated the genotoxic potentiality of saliva of Kwai-chewers of the tribal population of Meghalaya state of North-Eastern region of India in Chinese hamster ovary cells. The average age of onset of chewing among tribes was about 12 years. Thus in the period between onset of chewing and diagnosis of tumors, the oral mucosa was exposed for about 28,000 hours to Kwai and leaf extracts if we assume an average 12min per chewing period. Among kwai-chewers of 35 years of age and older, the frequency of oral carcinoma rises significantly. Therefore, the purpose of this study was to investigate the extent of DNA damage, delay in cell cycle kinetics and p53 expression in Kwai chewers in the tribal population of Meghalaya state of North-Eastern region of India and its correlation with endogenous GSH level.

Materials and methods:

Human peripheral blood lymphocytes:

Blood was collected from healthy donors (non-chewers and chewers) with age ranging from (24-70) years. For lymphocyte culture whole blood was used and cultures were set with 5-bromodeoxyuridine. For GSH estimation and levels of p53 protein detection, lymphocytes were isolated from heparinised whole blood on a Ficoll hypaque (FH) density gradient.

Selection of subjects:

On an average each Kwai weighs 10g and is cut into four pieces. Out of 37 donors, 27 individuals were chewing a mixture of Kwai, lime and betel leaf and 10 healthy individuals are non-chewers. The subjects were divided into the following three groups:

Non-chewers (NC): Individuals who have never chewed Kwai in any form were included in this category. Within 10 NC controls five were males and five were females.

Moderate chewers (MC): Ten individuals consuming around 40 to 55 grams of kwai with an average of 20 chews per day were considered as MC. Except for one who was a female, the rest were males.

Heavy chewers (HC): Seventeen individuals consuming around 100-120 grams of nut with an average of 40 chews per day were included in this category. The HC were mostly males except for two females.

All subjects were surveyed for personal information which included number of Kwai per day as well as life style information such as age, smoking history (if any), consumption of alcohol, duration of Kwai chewing. Most of the donors were non-smokers except for two or three in each category having mild smoking habit. It should be noted that most chewers swallow their saliva. All the donors had no viral disease or antibiotic therapy during the last 6 months. Informed consent was obtained from all the individuals studied before sample collection.

Culture was done from all the donors. 8 NC (all except donors 9 and 10) and 8 HC (Donors numbers were 1, 4, 5, 7, 8,11, 12, 15) were randomly selected for the determination of the level of p53 protein. The endogenous GSH level was estimated from each sample. The controls and chewers were pre-identified from the staff members of our university and nearby villages.

Table 2.1 *shows the age and chewing patterns of the samples studied.*

The procedure for culture setting, GSH estimation and western blot analysis has been described in Chapter 1. The preparation of metaphases, differential staining, scoring and statistical analyses have also been described in the previous chapter. However, here blood was used immediately after collection without any treatment. It should be noted here that for p53 protein level, 80μg of protein was loaded in each lane in order to obtain representable bands from differentiated lymphocytes.

RESULTS

(a) Levels of tGSH

The level of total GSH in the donors is shown in table 2.2, figure 2.1. In the control NC group, the levels of GSH ranged from 5.06-10.33 $\mu m/10^6$ cells with a mean value of 7.05 $\mu m/10^6$ cells. In MC, there was a tendency of depletion with GSH level ranging from 4.06-7.90 $\mu m/10^6$ cells with a mean value of 6.06 $\mu m/10^6$ cells. In HC, the levels of GSH ranged from 2.02-4.54 $\mu m/10^6$ cells with a mean value of 3.12 $\mu m/10^6$ cells. Thus it was observed that there was 14% and 56% reduction of GSH levels in MC and HC respectively when compared to NC controls. The reduction in HC is statistically significant. Not much change in GSH levels on the basis of their age and sex were seen, except for some females in the NC category which show slightly higher GSH values than male donors.

(b) Chromosomal Aberrations (CAs): The data for CAs is shown in table 2.3, figure 2.2. There was marginal increase in CAs frequency in most MC and HC as compared to non-chewers. The aberrations were mostly of the chromatid break type. Although the frequency of aberrant metaphases in HC is not significantly increased with respect to NC but it clearly shows a positive tendency of induction of aberrations in HC. In several HC samples the frequency of aberrant metaphases was 4 and 5% which is certainly higher than NC and MC. It is also worth noting that these HC are more than 40yrs of age which showed higher frequency of aberrant metaphases.

(c) Sister chromatid exchanges (SCEs): Table 2.4, figures 2.2 show the SCE frequency in the donors. In HC, there was significant increase in SCEs when compared to NC. 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. Figure 2.3 shows the distribution of SCEs per cell in the RBN-chewers and non-chewers. In MC the number of cells having 7-10 SCEs increased as compared to NC. In HC there was a considerable decrease in the frequency of cells having 0-3 SCEs and correspondingly significant increase in cells having 11-15 SCEs with respect to NC and MC. However, three HC samples showed 2,7 and 18% of cells having more than 15 SCEs. The dispersion analysis indicated that the distribution of SCEs in all the categories did not deviate from Poisson distribution.

(d) Cell cycle kinetics: Table 2.5, figure 2.4 shows that the percentage of M1 cells increased in both MC and HC. There was an increase by 6% in M1 cells in MC when compared to NC while in HC the increase was significant. The AGT value increased significantly in HC indicating delay in the progression of the cell cycle. The difference in AGT values between NC and MC is worth noting indicating that the timing of cell cycle was increased by 2hrs., however, for HC the AGT value was increased by 5 hours. In case of mitotic index scoring, the induction of delay is reflected in chewers than NC but between MC and HC there

was no difference with respect to mean mitotic index. However, individual MI value of HC indicates that around 50% samples showed 2 or less than 2% which was not so for MC and NC.

(e) p53 protein level

Representative results of Western blot analysis are illustrated in Figure 2.5. 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.

Table 2.1 Summary data on the age and chewing pattern among the samples studied.

Subjects	*Age (Yr.) [Range]*	*Frequency of areca nut chewing (times/day)*	*Duration of the habit (Years)*	*Amount of betel nut chewed (gm/day)*
NC ***(n=10)***	32 [24-50]	--------	------	--------
MC ***(n=10)***	30.5 [25-36]	21.2 ± 0.998	10 ± 1.00	52.3 ± 2.15
HC ***(n=17)***	39 [27-70]	47.65 ± 2.50	24 ± 3.06	120 ± 6.27

NC- Non-chewer, MC- Moderate Chewer, HC- Heavy Chewer

Table 2.2 Concentration of total GSH in PBLs of RBN chewers and non-chewers.

Donor #	*Type of chewer*	*Age (yrs.)*	*Sex*	*Total GSH (μmol / 10^{-6}cells)*	*Mean ± SEM (Reduction%)*
1	NC	24	M	5.31	
2		33	M	5.06	
3		33	M	6.41	
4		50	M	6.82	
5		37	M	5.71	
6		27	F	5.19	
7		33	F	7.01	
8		30	F	10.33	
9		25	F	9.70	
10		28	F	8.97	**7.05± 0.62**
1	MC	25	M	4.06	
2		27	M	4.98	
3		31	M	5.04	
4		26	M	7.90	
5		32	M	7.06	
6		29	M	4.36	
7		41	M	6.94	
8		28	M	6.48	
9		36	M	6.07	
10		30	F	7.68	**6.06± 0.44 (14%)**
1	HC	27	M	2.02	
2		29	M	3.15	
3		35	M	4.45	
4		46	M	2.67	
5		39	M	3.57	
6		32	M	3.76	
7		50	M	2.77	
8		33	M	3.65	
9		34	M	3.28	
10		29	M	2.90	
11		70	M	3.59	
12		27	M	2.02	
13		37	M	4.54	
14		49	M	2.63	
15		42	M	3.20	
16		28	F	2.52	
17		60	F	2.40	**3.12[a]± 0.18 (56%)**

[a] $p<0.001$, student's t-test compared to non-chewer value.

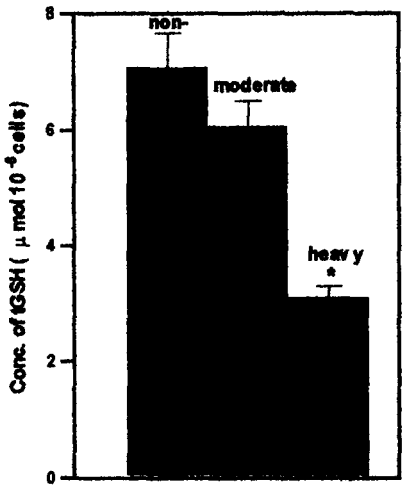


Fig: 21 Level of tGSH in PBLs of chewers and non-chewers. Each point and bar represent a mean±SEM value. *p<0.05 as compared with non-chewers.

Table 2.3 Induction of CAs in PBLs of RBN chewers and Non- chewers.

Donor #	*Type of chewer*	*Sex*	*Age (Yrs.)*	*Amt. of nut Pieces/day*	*TM*	*Abt.M(%)*	*Aberration (%) Chd.bk*
1	**NC**	M	24	----------	054	02	02
2		M	33		057	02	02
3		M	33		090	02	02
4		M	50		112	02	02
5		M	37		100	01	00
6		F	27		085	02	02
7		F	33		095	01	01
8		F	30		059	02	02
9		F	25		074	01	01
10		F	28		085	02	02
Mean ± SEM						**02±0.2**	**01±0.3**
1	**MC**	M	25	15-20	068	03	03
2		M	27	20-25	094	02	02
3		M	31	25-30	096	01	01
4		M	26	20-25	118	01	01
5		M	32	25-30	093	03	03
6		M	29	25-30	092	03	03
7		M	41	20-30	088	03	03
8		M	28	25-30	105	03	03
9		M	36	25-30	097	02	02
10		F	30	15-20	109	02	02
Mean ± SEM						**02±0.26**	**02±0.26**
1	**HC**	M	27	30-40	091	02	02
2		M	29	30-40	076	01	01
3		M	35	30-40	112	03	03
4		M	46	40-45	112	04	04
5		M	39	40-45	093	04	04
6		M	32	50-60	091	02	02
7		M	50	50-60	110	03	03
8		M	33	40-45	088	03	03
9		M	34	40	075	04	04
10		M	29	50-60	079	03	03
11		M	70	70-75	086	05	05
12		M	27	30-40	093	03	03
13		M	37	35-40	067	03	03
14		M	49	40-45	076	04	04
15		M	42	30-40	110	04	04
16		F	28	40	123	03	03
17		F	60	40-50	105	04	04
Mean ± SEM						**03±0.24**	**03±0.24**

TM-Total metaphases for aberration study, Abt.M- Aberrant Metaphases.

Table 2.4 Induction and distribution of SCEs in PBLs of RBN chewers and Non-chewers

Donor #	*Type of chewer*	*Cells scored*	*SCE/ cell*	*SCE /M(%)*					*H*
				0-3	*4-6*	*7-10*	*11-15*	*>15*	
1	NC	29	5.75	21	55	14	10	00	1.68$
2		34	6.12	12	44	41	03	00	0.82
3		32	5.16	16	66	16	03	00	0.73
4		32	5.54	16	59	25	00	00	1.06
5		39	5.94	13	49	38	00	00	0.66
6		55	5.88	13	53	31	04	00	0.80
7		34	5.90	03	62	35	00	00	0.85
8		33	5.21	24	48	24	03	00	1.17
9		37	4.62	32	49	16	03	00	1.16
10		32	5.47	19	50	28	03	00	0.88
Mean ± SEM			**5.56±0.14**	**17**	**54**	**27**	**03**	**00**	**0.98**
1	MC	47	6.40	13	38	45	04	00	0.92
2		38	4.42	39	37	24	00	00	1.33
3		36	5.76	14	50	36	00	00	0.85
4		42	5.48	21	50	29	00	00	0.71
5		35	6.86	03	49	40	06	03	1.14
6		27	6.48	07	37	56	00	00	0.59
7		31	5.10	16	61	19	03	00	0.93
8		32	5.97	16	44	38	03	00	0.88
9		35	6.26	17	29	54	00	00	0.93
10		36	6.83	06	33	61	00	00	0.62
Mean ± SEM			**5.96 ±0.25**	**15**	**43**	**40**	**02**	**00**	**0.89**
1	HC	26	7.12	08	38	42	12	00	1.24
2		47	7.51	09	36	36	17	02	1.73$
3		52	5.42	25	48	21	06	00	1.19
4		24	8.38	00	38	42	21	00	1.09
5		29	8.72	07	14	55	17	07	1.45
6		32	7.03	09	28	59	03	00	0.84
7		28	6.71	11	39	32	16	00	1.17
8		26	6.88	08	42	38	12	00	1.10
9		23	7.61	09	30	39	22	00	1.74$
10		32	6.34	09	47	38	06	00	1.18
11		24	7.58	00	42	38	21	00	1.40
12		25	6.60	08	32	56	04	00	0.72
13		44	11.02	05	11	30	36	18	1.65$
14		35	6.94	03	43	46	09	00	0.84
15		28	7.89	08	25	39	25	00	1.26
16		42	5.35	19	52	29	00	00	0.73
17		26	5.65	23	42	31	04	00	1.15
Mean ± SEM			**7.22[a]±0.33**	**09**	**36**	**39**	**14**	**02**	**1.20**

H: dispersion coefficient= variance/mean, $ significantly different at α= 0.05 from Poisson distribution; [a]$p<0.05$; student's t-test compared to non-chewer control

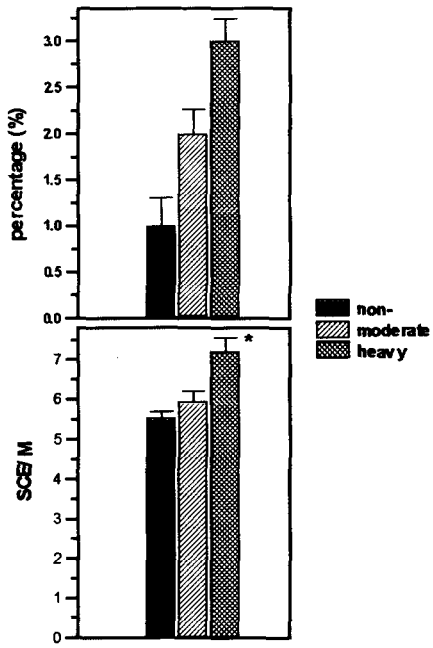


Fig: 2.2 Frequency of CAs and SCEs in PBLs of RBN-chewers and non-chewers. Each point and bar represents the mean±SEM value. *p<0.05 compared to non chewers.

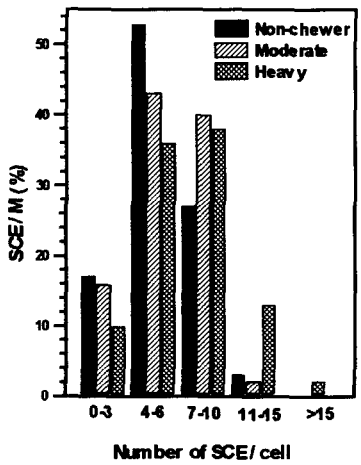


Fig:2.3 Frequency distribution of SCEs in PBLs of Non-, moderate, and heavy chewers.

Table 2.5 Cell cycle kinetics in RBN chewers and Non-chewers.

Donor #	Type of chewer	TM_1	Cells in %			AGT(H)	Mitotic Index	
			MI	M2	M3		TM_2/Total cells	(%)
1	NC	132	54	35	11	45.00	36/0968	3.72
2		129	52	35	14	44.17	32/1758	1.82
3		251	43	24	33	37.89	50/1322	3.78
4		257	47	20	33	38.71	45/1278	3.52
5		196	52	35	13	44.80	42/1102	3.81
6		242	46	45	09	44.44	69/1716	4.02
7		278	34	40	26	37.50	41/1380	2.97
8		142	42	47	11	42.86	26/1019	2.55
9		230	32	43	25	37.31	56/1582	3.54
10		145	60	34	10	48.09	36/1019	3.53
Mean ± SEM			**46±2.8**			**42.08±1.2**	**433/13144**	**3.33 ±0.2**
1	MC	234	41	41	19	40.45	41/1136	3.61
2		219	53	33	14	44.79	56/1458	3.84
3		244	45	39	17	41.86	16/1066	1.50
4		236	52	39	10	45.55	59/1680	3.51
5		156	61	32	07	49.32	30/1172	2.56
6		159	61	22	17	46.15	12/1101	1.09
7		146	63	27	10	48.98	34/1411	2.41
8		225	46	33	19	41.86	28/1120	2.50
9		193	51	31	19	42.86	43/1424	3.02
10		234	47	35	18	42.35	22/1038	2.12
Mean ± SEM			**52±2.4[b]**			**44.42±1.0**	**340[a]/12606**	**2.62(0.3**
1	HC	168	65	21	12	50.35	32/1358	2.36
2		243	32	30	38	34.92	77/1503	5.12
3		242	49	29	22	41.62	67/1704	3.93
4		163	70	23	07	52.55	41/0794	5.16
5		171	58	30	12	46.75	58/1309	4.43
6		156	58	33	08	48.00	33/1274	2.59
7		159	71	23	06	53.33	28/1280	2.19
8		138	67	25	08	51.06	22/1279	1.72
9		134	57	33	10	47.37	19/1098	1.73
10		149	55	31	14	45.28	48/1463	3.28
11		129	73	19	08	53.33	18/2069	0.87
12		154	64	23	14	48.00	28/1116	2.51
13		160	45	44	11	43.37	28/1538	1.82
14		146	52	32	16	43.90	21/1367	1.54
15		159	72	23	06	53.73	44/1334	3.30
16		232	55	31	14	45.27	18/1259	1.43
17		192	60	29	11	47.68	14/1157	1.21
Mean ± SEM			**59± 2.6[b]**			**47.44±1.2[#]**	**596[b]/22902**	**2.66±0.3**

TM1- Total metaphases for cell kinetics; TM2- Total metaphases for mitotic index, M.I-Mitotic index #p<0.01;student's t-test compared to non-chewer controls; [a]p<0.01,bp<0.001 2x2 contingency test compared to non chewer controls.

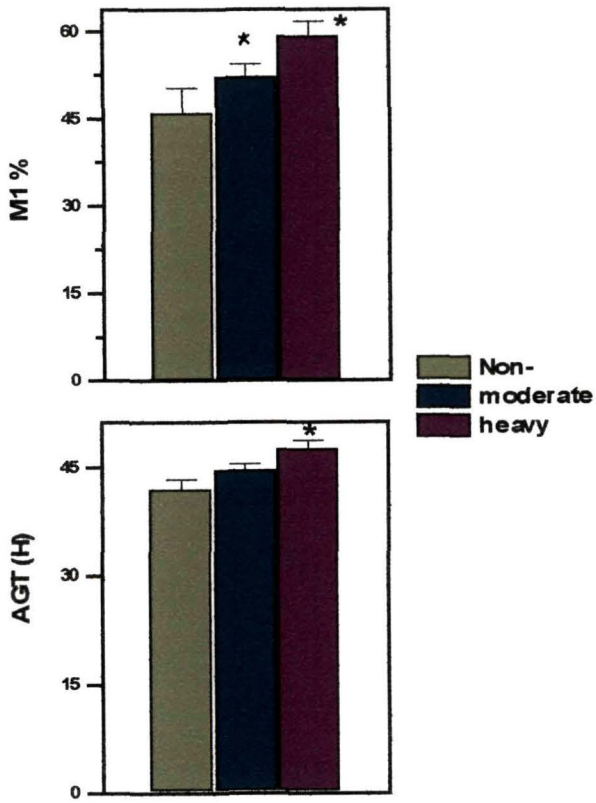


Fig: 2.4 Cell kinetics in chewers and non-chewers.
Each point and bar represents the mean±SEM value
*p<0.05 compared to non chewer control.

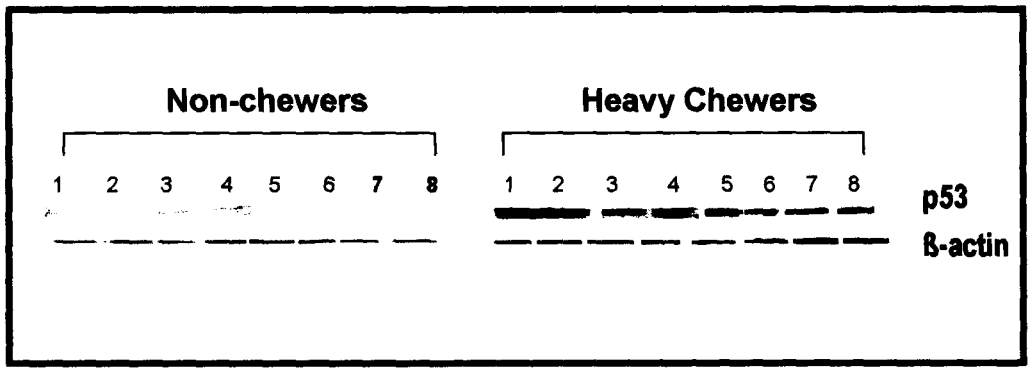


Fig. 2.5 **The level of p53 protein in blood lymphocytes of kwai-chewers and non-chewers**

Discussion

The present data show higher DNA damage, delay in cell kinetics, p53 expression and lower GSH-level in HC than NC and therefore these parameters could be a valid and reliable predictor of cancer occurrence among Kwai chewers. Considerable interindividual variations have been observed regarding the dose, duration of chewing Kwai and occurrence of the disease. In Taiwan, 80% of total deaths due to oral cancer has been reported to be associated with betel-chewing habit. Hence, a great deal of human morbidity and mortality can be prevented by watchful monitoring. In the present 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 peripheral blood lymphocytes (PBLs) in chewers not suffering from cancers. It has been reported that the frequency of SCEs in lymphocytes was elevated in BN chewers and oral cancer patients in comparison to non-chewer controls (Adhvaryu et al 1991). Moreover, it has been proposed that the CA frequency in PBLs may serve not only as a biomarker of mutagen exposure, but also as a biomarker of genetic damage of relevance for carcinogenic processes (Aitio et al 1988, Bonassi et al 2000). Therefore, in this study we analyzed 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 (Chagnati et al 1974, Buckton et al 1978).

Alkaloids and ploy phenols of the Kwai have been the main suspect for delivering carcinogenic chemicals to the masticators (IARC 1985, IARC 1987) however, the ingredients of betel-leaf also show the mutagenic, carcinogenic and gene convertable potentialities (Stich et al 1983). Due to the presence of lime in Kwai preparation, BN-chewers' saliva typically changes from neutral to an alkaline condition. Rosin (1984) noted that BN ingredients can release reactive oxygen species under alkaline conditions and Nair et al (1987) noted that H_2O_2 and

superoxide radicals are produced during autoxidation of BN-ploy phenols when the pH level is greater than 9.5. It is well evident that ROS are important in the initiation and promotion of cancer (Cerutti 1985). Another important component of BN and betel leaf is the transition metal ions such as Cu^{2+}, Mn^{++}, Fe^{2+} and Fe^{3+} promote the production of ROS by BN (Nair et al 1987). Therefore, it is clear that all the components generating from the Kwai mixture have the potentialities of cancer induction.

The present data show distinctly more DNA damage and less endogenous GSH-level in PBLs of HC than NC. BN extract has been shown to induce cytotoxicity, DNA strand breakage, DNA-protein cross-linkage and unscheduled DNA synthesis of oral fibroblasts and keratinocytes (Jeng et al 1994, Jeng et al 1999). 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 (Hoffman et al 1994). Biochemical studies have found that ARC and arecaidine react with thiol groups both *in vivo* and *in vitro* to produce cysteine 3-alkylation adducts (Nery 1971). This may explain the cause of the depletion of GSH in oral fibroblasts and keratinocytes (Jeng et al 1996) and buccal mucosa of betel-quid chewers (Wong et al 1994). Thus the depletion of cellular GSH by ARC may render the cells susceptible to potential further attack by other BN components or environmental toxicants. Therefore, it is plausible to believe that the target tissues like buccal, gingival and oral fibroblasts and keratinocytes of HC most likely have more damage than non-target tissues. Significant increase in breakage / exchanges was observed in the BQ chewing population compared to controls. There was also significant association between breakage / exchange frequencies and both duration of chewing and the number of quids used per day (Rupa and Eastmond 1997). The HC show appreciable increase in SCEs which shows that continuous exposure to Kwai is more damaging. The association between duration of Kwai chewing and SCEs is intriguing in that it indicates that continuous exposure of the cells to Kwai may have induced persistent alterations

in the lymphocytes. These observations were made in buccal mucosal cells also (the target tissue) where a significant increase in breakage / exchange was observed in chewing population compared to controls (Rupa and Eastmond 1997).

The cell kinetics of PBLs of HC in culture condition showed significantly slower progression than NC. It has been reported that BNE can decrease the clonal cell growth of buccal epithelial cells (Sundqvist et al 1989), gingival keratinocytes (Jeng et al 1999) and fibroblast cells (VanWyk et al 1994). Earlier ARC showed the ability of induction of delay in cell kinetics in mouse BMCs (Deb and Chatterjee 1998) and similar observation was made by others in human gingival fibroblasts (Jeng et al 1996) and also in skin fibroblasts (VanWyk et al 1995). It is known that ARC by losing only one of its methyl groups during metabolism (Boyland and Nery 1969) may bind nucleic acids and proteins (Nery 1971), and such a DNA adduct may interfere in cell proliferation. ARC and BN extract was shown to inhibit fibroblast growth and can also deplete cellular GSH (VanWyk et al 1995, Jeng et al 1994). In the present study HC showed low GSH level which could also be responsible for the delay since cellular GSH are important for cell proliferation (Jeng et al 1996).

By feeding Swiss albino mice with BN diet, lipid peroxidation, glutathione-S-transferase and cytochrome P450 activity were elevated in the liver with a concomitant GSH depletion (Singh and Rao 1995). Biochemical studies have shown that arecoline and arecaidine react with thiol groups both *in vivo* and *in vitro* to produce cysteine-3-alkylation adducts (Boyland and Nery 1969, Nery 1971). The extracellular addition of GSH and cysteine has been shown to prevent the ARC cytotoxicity to cultured OMF (oral mucosal fibroblasts) *in vitro*, although superoxide dismutase (SOD) and catalase lacked similar preventive effects. Therefore, present low GSH level in HC could also be due to interaction of RBN components with thiol groups and render the cells more vulnerable to

other reactive agents such as nitrosamines which will form from ARC in the cells within a few hours (Wenke and Hoffmann 1983).

The SCE elevation in PBLs observed in the present study are in accordance with the reports of others (Adhvaryu et al 1991, Adhvaryu et al 1986). The dispersion analysis indicated that the distribution of SCEs 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 DNA damaging agent that induces SCEs fit well with the Poisson distribution (Rainaldi and Mariani 1982). From the intracellular distribution of SCEs induced, there was an increase in the cells having 7-10 SCEs in MC and increase in cells having 11-15 SCEs in heavy chewers. However, one HC sample showed 18% cells having more than 15 SCEs per cell and its SCE distribution along with couple of others distribution deviated from Poisson distribution. Margolin and Shelby (1985) demonstrated the groups of individuals with non-significantly different SCE means could have significantly different SCE / cell dispersions. Some chemicals, either due to very limited distribution or to highly specific cell stage specificity, may induce a significant increase in dispersion in the absence of a significant increase in mean SCE frequency. This analysis, suggests that examining the distribution of SCEs, in addition to mean frequency, is a useful method for evaluating agent specific patterns in SCE induction (Tice et al 1989). The biological basis for this increasing hyperdispersion with increasing mean SCE frequency in some HC samples has not been resolved, and it has been proposed that cell to cell differences in induced damage, proliferative capacity, cell stage sensitivity, DNA-repair activity could modulate the distribution of SCE observed among exposed cell populations (Sasaki 1982, Shafer 1982). From the present result it is right to speculate that the induction of SCEs is due to DNA-damage and such damage will be several degree more for the target cells. However, it seems that such DNA lesions could not lead to chromosomal aberrations since the induction of CAs

was also marginally increased in HC than NC which could also be explained on the basis of the clastogenic potential of Kwai components. In the present study, the HC were older (mean age 41yrs) than the other groups (mean age for NC and MC are 32 and 30 years) and also having longer duration of chewing habit. However, reports regarding the effect of age and sex on the frequencies of SCEs and CAs were contradictory (Margolin and Shelby 1985, Husum et al 1986) and also have no significant influence on the frequency of cytogenetic endpoints in question (Adhvaryu et al 1991, Bonassi et al 2000). Here, due to the small sample size in each group, the evaluation of the effect of these confounding factors will not be meaningful.

The present data indicate higher induction of DNA damage and delay in cell kinetics in HC. 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 (Hall et al 1993, Zhan et al 1994), by blocking the damaged cells at the G1 checkpoint and then presumably allowing time for DNA repair (Smith et al 1995). Thus the present observed increase in delay 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 (Kastan et al 1991). Increased expression of p53 protein has also been reported in head and neck squamous cell carcinoma (Chiba et al 1996, Ahomadegbe et al 1995). Moreover, elevated levels of p53 protein have been observed not only in oral squamous cell carcinomas but also in oral dysplastic lesions, suggesting that p53 alteration is an early event in oral oncogenesis (Kaur et al 1998). 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 (Shin et al 1994). 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 which was proposed by earlier workers (Shin et al 1994, Bennet et al 1992). Higher frequencies of mutations in the p53 gene or increased expression of p53 protein has also been reported in head and neck SCC in Japan (Sakai and Tsuchida 1992, Chiba et al 1996) and western countries (Hollstein et al 1990, Somers et al 1992, Caemano et al 1993, Shin et al 1994). Thus continuous exposure of Kwai will induce much higher frequencies of DNA damage, which could ultimately lead to destruction of some important genes, including p53 and thus cells with DNA damage might proliferate ultimately leading to neoplastic transformation. It is indeed p53 gene is the most frequently mutated gene in human oral cancers (Thomas and MacLennan 1992).

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 (McMichael and Hall 1997). This p53 expression can be taken as a specific marker of malignancy. Hall et al 1991 stated that the presence of p53 immunoreactivity can be used to infer neoplastic growth. The rapid degradation of p53 in non- transformed cells occurs via a non-lysosomal ATP-dependent proteolytic pathway (Ciechanover et al 1991). The retarded degradation and nuclear accumulation conferred upon abnormal p53 proteins is a reflection of elevated stability and self-aggregation of the mutated molecules (Halevy et al 1989). The p53 overexpression might be indicative of the presence of a p53 mutation. This overexpression of p53 may be a biologic marker indicative of improved survival potential and does not affect the prognosis as has been reported by some investigators (Brambilla et al 1993, Sauter et al 1992). It can be said that this p53 expression may be an early event in oral tumorigenesis, resulting in poor prognosis. These may be used in identifying a subset of normal Kwai chewers with poor prognosis in the high risk population. In depth studies on

larger populations may yield important predictive information, thereby reducing morbidity, mortality and the cost of medical treatment for people belonging to lower socio-economic groups. So people can be made aware before cancer has really occurred. Hitherto, only cytogenetic biomarkers have been validated (Rothman et al 1995). From this studies 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. Because the presence, quantity and pattern of expression of the biomarkers should correlate to the probability of malignant transformation of a cell or tissue (Wogan 1992). Jeng et al 1994 suggested that increasing dietary intake of GSH rich foods or dietary supplementation with GSH may have chemopreventive potential to reduce BN associated oral lesion. But, though this may block nitrosation reactions *in vivo* in Kwai chewers, cessation of Kwai chewing habit is the only safe way for an efficient reduction of cancer risk.

CHAPTER III

ARE BETEL NUT CHEWERS SENSITIVE TO ANY OTHER MUTAGEN?

Literature Review

Molecular epidemiological studies have provided evidence that individual susceptibility to environmental carcinogens must result from several host factors including the genotype or phenotype of carcinogen-activating and detoxifying enzymes and DNA repair enzymes. And so there is an imperative need for molecular markers that can predict whether a premalignant lesion will develop into an aggressive or metastasizing tumour. The development of head and neck cancer may depend upon the interaction between the host susceptibility factors and environmental carcinogens. Tobacco and alcohol have been implicated as the etiologic agents in ~80% of individuals (Blot et al 1998). Host susceptibility factors are now being elucidated and may include abnormalities in carcinogen metabolism, factors linked to blood group antigen expression, as well as deficiencies in DNA repair. One of the more frequently identified genetic alterations in head and neck cancers involve the short arm of chromosome 3 in which chromosomal rearrangements and deletions predominate and this damage may be influenced by the interaction between carcinogen exposures and host susceptibility factors. One such factor within head and neck cancer patients may be reflected in the mutagen sensitivity assay (Hsu et al 1989, Schantz and Hsu 1989, Cloos et al 1999). The assay makes use of peripheral blood lymphocytes in order to test for Bleomycin (BLM) induced chromosomal breakage *in vitro*. BLM induces chromosomal damage through the generation of free radical oxygen, and thus, is reflective of one measure of tobacco / betel quid induced damage. Studies have demonstrated that head and neck cancer patients may be abnormally sensitive to BLM induced chromosomal damage as compared with age and sex matched healthy controls (Spitz et al 1989, Spitz et al 1993, Cloos et al 1994, Schantz et al 1997, Cloos et al 1996, Li et al 1994, Pandita and Hittelman 1995, Schantz et al 1990). Furthermore risk assessments in this studies suggest an interaction between carcinogen exposure and mutagen

sensitive measures, risk estimates being highest in those individuals who both consume tobacco and express sensitivity to free radical damage *in vitro* (Spitz et al 1993, Schantz et al 1997). The basis of this mutagen sensitivity may reflect an underlying DNA repair deficiency or factors which control susceptibility to initial clastogenic influences (Pandita and Hittelman 1995). Dave et al 1994, suggested that chromatid break sites induced by BLM *in vitro* were not random but rather were predetermined by host-susceptibility factors as well as specific mechanisms related to free radical oxygen chromatid interactions. In support of this hypothesis, lymphocytes obtained from head and neck cancer patients more frequently demonstrated breaks on the short arm of chromosome 3 following BLM exposure *in vitro* than similarly treated lymphocytes from either healthy controls or patients with melanoma. For cancers of the respiratory and upper digestive tracts, traditional epidemiology has identified smoking and alcohol intake as major risk factors (Maier et al 1992, Baron et al 1993). Molecular epidemiology is a relatively new approach that incorporates individual biomarkers for cancer risk assessments in populations. An interaction between exposure to carcinogens and susceptibility factors was found to determine cancer risk. Susceptibility biomarkers have been reported, for instance, on carcinogen detoxification, carcinogen activation, and formation of DNA adducts (Spivack et al 1997, Vinies et al 1994, Kato et al 1995).

There is increased interest in using biological markers to monitor populations for identification of excessive toxicants. The ultimate goal is to use these biomarker data to indicate early disease and to predict increased risk for development of long-term health consequences. The choice of biomarkers for population studies will therefore determine the usefulness of the results in public health. Chromosome aberration is one of the most extensively used biomarkers for population monitoring. Unlike most other biomarkers, existence of chromosome aberrations has been shown to be associated with health effects. For example, chromosome aberration is frequently used as an internal biological dosimeter for

exposure to ionizing radiations (Awa et al 1971, Bauchinger 1984) and the dose-response relationship of chromosome aberration to exposure is similar to that for leukemia mortality (Bender and Wong 1982). In a prospective cohort study, populations with increased chromosomal aberrations have higher leukemia mortality than those with lower chromosome aberrations (Sorsa et al 1990, Hagmar et al 1994). Therefore this biomarker can be used to predict health consequences.

Susceptibility to bleomycin-induced chromatid breaks in cultured peripheral blood lymphocytes may reflect the way a person deals with carcinogenic challenges. This susceptibility (also called as mutagen sensitivity) has been found to be increased in patients with environmentally related cancers, including cancers of the head and neck, lung and colon, and, in combination with carcinogenic exposure, this susceptibility can greater influence cancer risk (Cloos et al 1999).

The drug bleomycin (BLM) is a mixture of closely related glycopeptide antibiotics, primarily bleomycins A_2 and B_2 isolated from *Streptomyces verticillus* (Povirk and Austin 1991, Burger 1998). BLM has found use in the treatment of cancers of the head and neck, squamous cell carcinomas, testicular cancer, and some lymphomas (Hay et al 1991, Stubbe et al 1996). BLM is said to be radiomimetic because its induction of genetic damage resembles that of ionising radiation. The primary features are S-phase independence and the induction of chromosome type aberrations in G_0 cells (Povirk and Austin 1991). Treatments of G_0 lymphocytes with BLM causes dose dependent increases in the frequency of chromosomal aberrations (Dresp et al 1978) and micronuclei (Hoffmann et al 1993a). As in the case of indirect action of radiation, BLM is also known to induce DNA breaks through the production of free radicals (Sausville et al 1976, Takeshita et al 1978).

To cause DNA damage, BLM must associate with DNA and be activated (Povirk and Austin 1991, Stubbe et al 1996, Burger 1998). The initial step is the binding of Fe^{+2}-BLM to DNA by hydrophobic and ionic interactions mediated by its

bithiazole moiety and C-terminus (Kane et al 1994). Activation occurred through addition of oxygen to Fe^{+2}-BLM, followed by a one electron reduction (Povirk 1996, Stubbe et al 1996) giving rise to a species called "activated bleomycin" whose probable structure is a ferric hydroperoxide (HOO- Fe^{+3}-BLM) (Stubbe et al 1996, Burger 1998). The metal binding portion of BLM which determines the sequence selectivity of strand scission, seems to be oriented in the minor groove of DNA (Kane et al 1994) where activated BLM specifically abstracts a hydrogen from the 4' position of deoxyribose, forming a free radical (Burger 1998). The addition of oxygen to the free radical at 4' gives rise to a peroxyl radical whose decomposition causes the release of a base propenal and a strand break with 5'-phosphate and 3'-phophoglycolate ends (Povirk and Austin 1991). After a single-strand break occurs at a primary cleavage site, the same molecule of BLM is apparently reactivated in situ and cleaves the complementary strand, resulting in a blunt-ended double-strand break (Stubbe et al 1996, Charles and Povirk 1998). Potentiation of the genotoxic activity of BLM with GSH has been observed (Chatterjee et al 1989). This could be due to GSH acting as a reducing agent either in reactivating oxidised Fe^{+3}-BLM to reduced Fe^{+2}-BLM or alternatively due to its binding directly with the DNA and altering the helical structure, thereby enhancing BLM binding itself (Chatterjee et al 1989). GSH acts by reducing the oxidized BLM, thus reactivating the spent BLM for further production of radicals causing more DNA breaks. Depletion of endogenous GSH by BSO reduced the clastogenic actions of BLM, whereas elevation of endogenous GSH by GSH and GSH-ester potentiate the cytotoxicity of BLM (Chattopadhyay et al 1997).

Thus an attempt has been made for mutagen sensitivity assay of the RBN-chewers since such assay may reflect either an underlying DNA repair deficiency or factors which control susceptibilty to initial clastogenic influences (Pandita and Hittelman 1995). As a cell source, peripheral blood lymphocytes were used since these are easily available and can be cultured for a few days from whole blood samples.

Materials and methods:

Materials: Human peripheral blood from 7 Non-chewers (Donor numbers 1 to 7) and 5 Heavy chewers (Donor numbers 4, 5, 7, 14, 15) of Kwai were selected randomly. This has been described in details in previous chapter.

Reagents:

Bleomycin sulphate (BLM, Biochem Pharmaceutical Industries, Mumbai, India): One vial of commercially available "Bleochem" used in this study, contains 15 mg potency of bleomycin sulphate. Stock solution of the concentration, 3000μg ml^{-1} was prepared by dissolving in 5 ml distilled water. Fresh working solution of the concentration 1000μg ml^{-1} was prepared in RPMI 1640 medium. From here the required amount was added to each culture so that the concentration is 30μgml^{-1} of culture.

The other reagents used for culture are same as described in the previous chapters.

Culture procedure: The standard lymphocyte culture as described earlier was followed, however with slight modifications. No BudR was added. At 67h of incubation, cultures were treated with BLM (30μg ml^{-1}) 5h before cell-harvesting, ensuring that damage induced in the late S and G_2 phases of the cell-cycle could be evaluated at metaphase. Colcemid was added 3h prior to harvesting to induce mitotic arrest. For each subject duplicate cultures were used.

Preparation of metaphases: The conventional cell harvesting procedure was followed. The slides were stained with 3% Giemsa and mounted in DPX.

Scoring and statistical analysis: Only chromosome aberrations were studied. Aberrations were scored as chromatid breaks and deletions. At least 100 well spread metaphases were examined from coded slides. Data were subjected to statistical analysis using the 2x2 contingency test for the frequency of aberrant metaphases and simple χ^2-test for aberration.

Results

Microphotographs representing different types of aberrations induced by BLM in NC and HC are depicted in figure 3.1.

Table 3.1, 3.2 and figure 3.2 shows the frequency of chromosomal aberrations in NC and HC. The range of the frequency of aberrant metaphases was 17 to 24% with a mean of 20% after exposing the G_2 lymphocytes to BLM in NC whereas in HC the range was 20 to 36% with a mean of 28%. Both the frequency of BLM induced chromatid break and deletions were increased in HC, however the extent of increase with respect to deletion was significant. The range of the frequency of chromatid break was 10 to 37% in NC whereas in HC the range was 17 to 54%. The frequency of deletions in NC was 10 to 27% whereas in HC the frequency was appreciably high ranging from 24 to 46%. Regarding the distribution of aberrations per cell it is clear from table 3.3, figure 3.3 that the number of cells having 4 or more than 4 aberrations were much higher in HC than in NC. In NC number of cells having 1 or 2 aberrations was more than cells having 3 or 4 aberrations per cell.

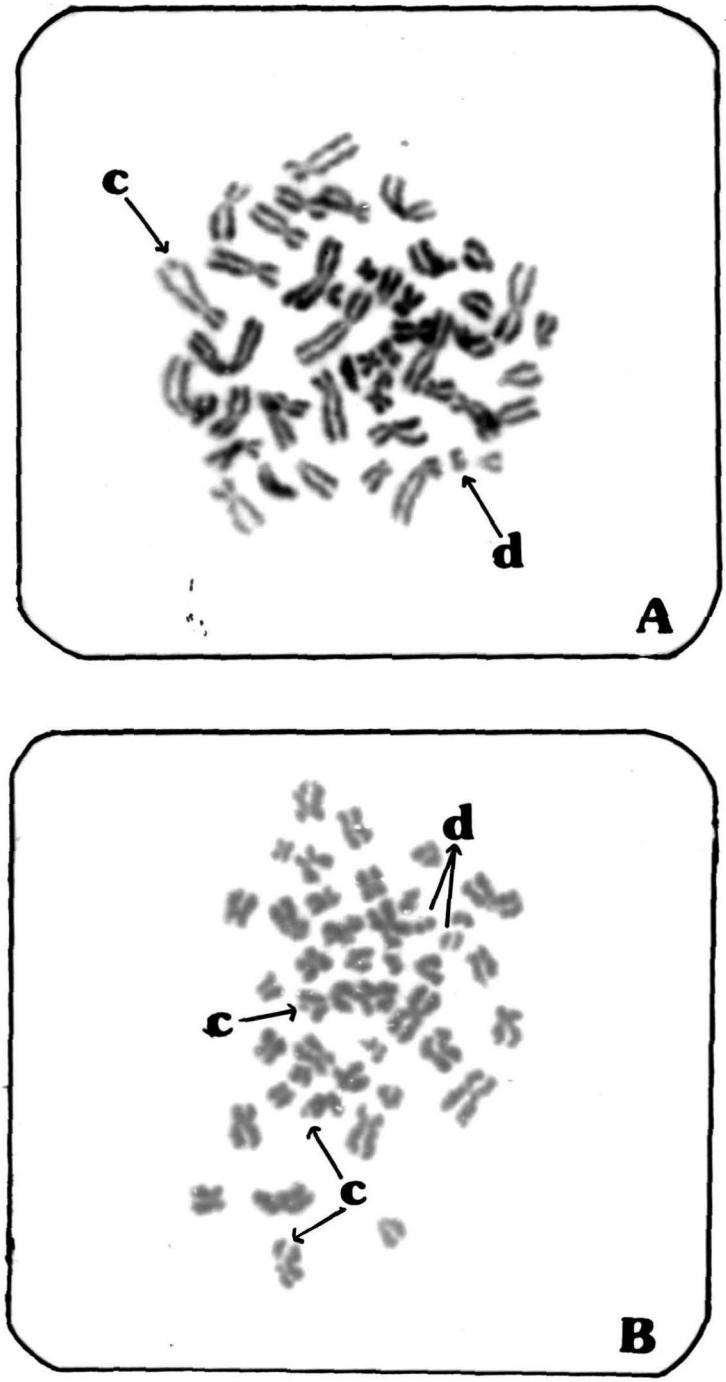


Fig 3.1: Microphotographs showing Bleomycin-induced aberrations in peripheral blood lymphocytes of non- and heavy chewers. Chromatid break (c), Deletion (d).
A. Non-chewer.
B. Heavy-chewer.

Table 3.1 Induction of CAs in PBLs of RBN chewers and non-chewers with BLM (30 μg ml^{-1})

Donor# /Age	*Type of Chewer*	*Experimental Condition*	*TM*	*Abt.M (%)*	*Chd.bk (%)*	*Del (%)*
1 / 24	NC	untreated	120	02	02	00
		BLM	105	17	10	10
2 / 33		untreated	107	01	01	00
		BLM	126	19	13	21
3 / 33		Untreated	132	02	02	00
		BLM	118	18	19	19
4 / 50		Untreated	145	02	02	00
		BLM	113	24	37	27
5 / 37		Untreated	143	01	01	00
		BLM	075	23	25	25
6 / 27		Untreated	141	01	01	00
		BLM	099	17	21	19
7 / 33		Untreated	197	02	02	00
		BLM	127	20	22	24
4 / 46	HC	untreated	100	04	04	01
		BLM	134	29	20	39
5 / 39		Untreated	120	04	04	00
		BLM	123	20	19	24
7 / 50		Untreated	079	05	05	00
		BLM	125	24	17	41
14 / 49		Untreated	134	04	04	00
		BLM	108	31	28	36
15 / 42		Untreated	120	05	05	00
		BLM	085	36	54	46

TM-Total Metaphases for aberration study, Abt.M- Aberrant metaphases,

Table 3.2 Pooled data on the induction of CAs in RBN chewers and non-chewers with BLM (30μg ml^{-1})

Donor #	*Type of Chewer*	*Experimental condition*	*TM*	*Abt.M(%) ±SEM*	*Chd.bk(%) ±SEM*	*Del(%) ±SEM*
07	NC	Untreated	985	02±0.2	02±0.2	00
		BLM	763	20±1.1	21±3.3	21±2.1
05	HC	Untreated	553	04±0.2	04±0.2	00
		BLM	575	28±2.8[a]	28±6.9	37±3.7[b]

[a]$p<0.001$; 2X2 contingency χ^2 test compared to non-chewer controls.
[b]$p<0.001$; χ^2 test compared to non-chewer controls

Table 3.3 Frequency distribution of Aberrations in BLM treated lymphocytes of RBN chewers and Non- chewers

Donor #	*Type of Chewer*	*Experimental Condition*	*Abt.M (%)*	*Aberration /cell (%)*				
				1	*2*	*3*	*4*	*>4*
1	NC	BLM	17	61	22	11	06	00
2			19	42	38	20	00	00
3			18	48	14	23	05	10
4			24	26	33	22	04	15
5			23	12	53	29	06	00
6			17	35	41	18	06	00
7			20	52	44	04	00	00
Mean				**39**	**35**	**18**	**04**	**04**
4	HC	BLM	29	23	21	20	18	18
5			20	48	24	04	16	08
7			24	37	29	20	07	07
14			31	32	29	18	12	09
15			36	23	23	16	19	19
Mean				**33**	**25**	**16**	**14**	**12**

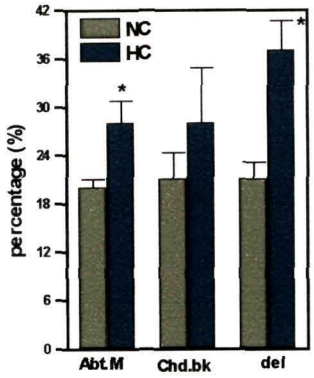
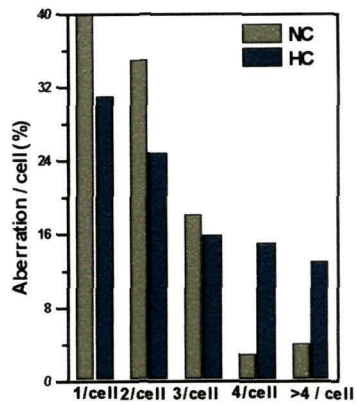


Fig: 3.2 Induction of aberrations by BLM in PBLs of RBN--chewers and non-chewers. Each point and bar represents a mean ±SEM value. *p<0.001 compared to non chewer control.



Fig; 3.3 Frequency distribution of aberrations induced by BLM in PBLs of non-and heavy chewers.

Discussion:

The present study is carried out to analyze the influence of BLM treatment on the induction of chromatid break in G_2 phase of PBLs in NC and HC to determine the individual susceptibility to carcinogenic assaults. For environmentally related cancers, such as colon cancer, lung cancer and head and neck squamous cell carcinoma (HNSCC), the biologic relevance of this marker has been well established (Hsu et al 1989, Scott et al 1994). It was shown earlier that an increase in the mean level of chromatid breaks per cell in cancer patients compared with healthy control persons (Hsu et al 1989, Scott et al 1994, Cloos et al 1994). BLM induces chromosomal damage 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 4 or more than 4 breaks were increased in HC. The relationship between a high susceptibility for the chromatid breaks and the development of environmentally related cancers have been established in retrospective (Hsu et al 1989) and a limited number of prospective studies (Spitz et al 1994, Schantz et al 2000). 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*. The great refinement of cancer risk assessment by use of the susceptibility to bleomycin-induced chromatid breaks indicated the importance of this biomarker. The phenotypes, mean chromatid breaks per cell, can be valuable determinant because it may be indicative of the cancer prone phenotype and can be used as an endpoint in these studies. Susceptibility to BLM induced chromatid breaks in cultured PBLs may reflect the way a person deals with carcinogenic challenges and it has the advantage that it can be assessed before the cancer has occurred. Avoidance of exposure to environmental and occupational carcinogens, especially in sensitive persons,

may then become an important factor in the prevention of cancer (Cinciripini et al 1997).

The basis of this mutagen sensitivity may reflect either an underlying DNA repair deficiency or factors which control susceptibility to initial clastogenic influences (Pandita and Hitelman . 1995). Dave et al 1994, suggested that chromatid break sites induced by bleomycin *in vitro* were not random but rather were predetermined by both host susceptibility factors as well as specific mechanisms related to free radical oxygen-chromatid interactions. It can be said that mutagen sensitivity is not an independent risk factor but a constitutional factor, which reflects the way in which genotoxic compounds are dealt with, and is thereby directly related to cancer risk.

It has been reported that the frequency of CAs by BLM enhanced significantly in the presence of GSH (Chatterjee et al 1989). However, BSO mediated GSH-depletion reduced the clastogenic action of BLM (Chattopadhyay et al 1997). Such reduction in the effect of BLM in GSH depleted cells could be attributed due to the failure of reactivation of the oxidised BLM by reducing agent GSH which is present endogenously. Radicals which are generated due to reduction of oxidised BLM by the increased level of cellular GSH could be responsible for the increasing frequency of deletion and chromatid breaks. In the present study, we found that the endogenous GSH level is reduced in HC than NC, however, the BLM induced more damage in HC than NC. Therefore, it seems that BLM-induced higher DNA damage in HC which could be due to inefficient DNA-repair and antioxidant machinery in addition to other cellular factors might play a role. There could be changes in the DNA repair machinery as has been reported earlier (Au et al 1995). Carcinogenesis is a multi step process involving multiple and sequential genetic alterations. It has been proposed that an initial step for the development of cancer involves the induction of a mutator phenotype (Loeb 1991). Recent evidence suggests that the phenomenon of mutator phenotype leading to genetic instability may be caused by DNA-repair deficiency (Loeb

1994, Modrich 1994), this repair deficiency may be caused by exposure to environmental toxicants. For e.g. tire-storage workers exposed to nitroso-compounds and hospital personnel exposed to antineoplastic drugs were deficient in methylguanine alkyl-transferase activities (Sagher et al 1989, Oesch and Klein 1992). Abnormal repair synthesis was documented in lymphocytes of cigarette smokers and drug addicts when their cells were challenged with UV light or chemicals (Madden et al 1979, Celotti et al 1989, Mayer et al 1991).

The assumption is that lymphocytes from toxicant exposed individuals will have DNA repair deficiency due to modification of DNA / proteins (e.g. adducts) or mutation of DNA repair genes and will make more mistakes in repairing the DNA strand breaks than lymphocytes from controls. The significance of DNA repair deficiency in the carcinogenesis process is that such abnormality causes multiple and sequential genetic alteration in cells, thus promoting the evolution of genetic change that are relevant to the development of cancer. Although the mechanisms for the abnormal DNA repair are not known, the abnormality may be caused by blockage of repair processes on DNA (eg adducts) or by mutation of genes that code for DNA repair enzymes (Au 1993). Because the DNA repair process involves multiple enzymes, it represents an enormous target for insult by toxicants. The abnormal DNA repair response will cause cells from affected individuals to make more mistakes in the repair of DNA damage, especially from further exposure to mutagens. The abnormalities may cause these populations to have increased health risk (Au et al 1996). The aberrations were more in HC and this will serve as an important biomarker to monitor populations for identification of excessive exposure to environmental toxicants. The idea of a casual association between CAs and cancer risk is based on the concept that genetic damage in lymphocytes reflects similar damage in cells undergoing carcinogenesis (Bonassi et al 2000). The ultimate goal is to use these biomarker data to indicate early disease and to predict increased risk for development of long-term health consequences. Based on these data, which requires

confirmation by a larger study, identification of high-risk subjects could now be explored to advise them on cessation of the habit before development of clinical symptoms of cancer. Because alterations of DNA repair genes, which are critical for maintaining DNA integrity can increase cancer susceptibility by increasing genomic instability. Excessive exposure to RBN could cause more DNA damage which might lead to abnormality in DNA repair, and genetic instability.

SUMMARY

Betel nut (BN) or areca nut is the hard. edible, endosperm of the palm *Areca catechu* Linn (palmaceae) which grows throughout south and south east Asia and in several pacific ocean Islands. The North-East Indian variety of BN is raw, wet and unprocessed (RBN, locally known as **Kwai** by the Khasi tribe of the North-Eastern region of India) consumed with betel leaf and slaked lime. The constituents of this nut show higher alkaloids, polyphenols and tannins as compared to the dry one. There are strong indications for a casual association between BN or quid chewing habit and oral mucosal diseases such as leukoplakia, oral submucous fibrosis and oral cancer. BN-extract (BNE) can induce DNA strand breaks, sister chromatid exchanges (SCEs) and micronuclei in various kinds of cells. The frequency of lymphocytic SCE was elevated in BN chewers and oral cancer patients in comparison to non-chewers.

Reduced glutathione (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 and various toxic effects of xenobiotics but not against radiomimetic drugs like bleomycin (BLM). Arecoline (ARC, an alkaloid of betel nut) induced chromosomal aberrations (CAs) in mice is enhanced by buthionine sulfoximine (BSO), a glutathione synthesis inhibitor and the genotoxic effect of ARC was reduced when it was administered with N-acetyl-L-cysteine (NAC). Therefore, the present study was undertaken to determine the genotoxic effect of RBNE with respect to endogenous GSH level. The disturbances in the antioxidant systems might be useful indicators of the susceptibility of subjects to free radical damage. Intracellular GSH status appears to be sensitive indicator of the cells' overall health and of its ability to resist toxic challenge. Two types of extract were chosen for this study- Aqueous extract of betel nut (AEBN) and Acetic acid extract of betel nut (AAEBN)

Tumor suppressor gene p53 mutations are the most common genetic abnormalities found in human cancers, especially in the development of head and neck cancer. Elevated levels of p53 protein have been observed not only in

oral squamous cell carcinoma but also in oral dysplastic lesions, suggesting that p53 alteration is an early event in oral carcinogenesis. 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. Another important purpose of this study was to investigate the extent of DNA damage, delay in cell kinetics and p53 expression in Kwai chewers in the tribal population of Meghalaya state of North-eastern region of India and its correlation with endogenous GSH level. We have made an attempt to see the level of p53 protein in mouse *in vivo* after RBNE treatment.

The development of head and neck cancer may depend upon the interaction between host susceptibility factors and environmental carcinogens. Studies by using the mutagen sensitive assay have demonstrated that head and neck cancer patients may be abnormally sensitive to BLM induced chromosomal damage as compared with age and sex matched healthy controls. The assay makes use of peripheral blood lymphocytes in order to test for BLM induced chromosomal damage through the generation of free radical oxygen, and thus is reflective of one measure of tobacco / BN induced damage. The basis of this mutagen sensitivity may reflect an underlying DNA repair deficiency or factors which control susceptibility to initial clastogenic influences. Risk assessments in these studies suggest an interaction between carcinogenic exposure and mutagen sensitive measures, risk estimates being higher in those individuals who both consume Kwai and express sensitivity to free radical damage in vitro. So in addition to these, 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.

Western blot analysis to determine the p53 protein levels in BN chewers and non-chewers and mutagen sensitivity assay in them were also performed.

The important aspect of this investigation is that the genotoxic effect of BN was studied in the non-target tissue (HPBLs) and it is known that genetic damage in lymphocytes reflects similar damage in cells undergoing carcinogenesis. From this study the following are the major observations that were made:

➢ The RBNE is genotoxic and the endogenous glutathione (GSH) level could influence its effect. The depletion of GSH did not have much influence on CAs but SCEs and delay in cell proliferation were significantly enhanced. The level of p53 protein appreciably increased after treatment with RNBE for longer duration.

➢The population study carried out in RBN chewers showed that the amount of DNA-lesions was increased in Heavy chewers (HC) than Moderate chewers (MC) and Non chewers (NC). CAs also showed a positive tendency of increase in HC. Significant delay in cell cycle progression was seen in HC and the GSH level also significantly reduced in HC. Such increased DNA-damage could arrest the cells at G1 checkpoint presumably allowing time for DNA-repair. This speculation is consolidated after observing an increased level of p53 protein in HC than NC.

➢From the mutagen sensitivity assay performed using bleomycin (BLM), it was observed that the frequency of CAs significantly enhanced in HC than NC. It is thus evident that HC are more susceptible to free radical damage or to damage by other toxicant. This reflects either an underlying DNA repair deficiency in BN chewers or factors which control susceptibility to initial clastogenic influences.
Thus, from the present investigation, it is clear that continuous and chronic exposure to BN causes more DNA damage and delay in cell cycle. The depletion of cellular GSH may render the cells susceptible to potential further attack by

other BN components. Such continuous exposure may attack the already present DNA damaged cells or cells which has just repaired and thereby activate the repair machinery for further action. This repeated repair process may lead to error-prone repair and ultimately leading to altered gene expression. Thus the endogenous GSH level and p53 protein expression serve as important biomarkers of DNA damage in RBN chewers and may be useful to assess and control the high-risk population of long-term health outcomes associated with exposure to xenobiotics.

REFERENCES

Adhvaryu, S.G., Trivedi, A.H., Jani K.H., Vyas R.C. and Dave, B.J. (1986) Genotoxic effects of ketamine on CHO cells. ***Archives of Toxicology,* 59**, 124-125.

Adhvaryu, S.G., Dave, B.J. and Trivedi, A.H. (1991) Cytogenetic surveillance of tobacco-areca nut (mava) chewers, including patients with oral cancer and premalignant conditions. ***Mutation Research,* 261**, 41-49.

Ahomadegbe, J.C., Barrios, M., Fogel, S., Bihan, M.L.L, Douc-Rasy, S., Duvillard, P., Armand, J.P. and Riou, G. (1995) High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumors and metastasis: absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. ***Oncogene,* 10**, 1217-1227.

Aitio, A. et al. (1988) eds. Indicators for assessing exposure and biological effects of genotoxic chemicals. Consensus and technical reports. Commission of the European Communities. Interational Programme on Chemical Safety (UNEP- ILO- WHO), ***World Health Organisation*** Regional Office for Europe, Institute of Occupational health, Finland.

Albertini, R.J. (1998) The use and interpretation of biomarkers of environmentally genotoxicity in humans. ***Biotherapy,* 11**, 155-167.

Allred, D.C., Clark, G.M., Elledge, R., Fuqua, S.A.W., Brown, R.W., Chamness, G.C., Osborne, C.K. and McGuire, W.L. (1993) Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node negative breast cancer. ***Journal of the National Cancer Institute,* 85**, 200-206.

Amstad, P. and Cerutti, P. (1990) Genetic modulation of the cellular antioxidant defense capacity. ***Environmental Health Perspectives,* 88**, 77-82.

Anderson, M.E., Naganuma, A. and Meister, A. (1990) Protection against cisplatin toxicity by administration of glutathione ester. ***FASEB Journal,* 4**, 3251-3255.

Arjungi, K.N. (1976): Areca nut: A review. ***Arzneimittelforschung,* 26**, 951-956.

Arrick, B.A., Nathan, C.F., Griffith, O.W. and Cohen, Z.A. (1982) Glutathione depletion sensitizes tumor cells to oxidative cytolysis. ***Journal of Biological Chemistry,* 257**, 1231-1237.

Ashby, J. (1992) Use of short-term tests in determining the genotoxicty or nongenotoxicity of chemicals, in: H. Vainio, P.N Magee, D.B. McGregor, A.J McMichael (Eds.), Mechanisms of carcinogenesis in Risk Identification (IARC Scientific Publication No. 116), ***International Agency for Research on Cancer,*** Lyon, pp. 135- 164.

Au, W.W. (1993) Abnormal chromosome repair and risk of developing cancer. ***Environmental Health Perspectives,* 101** (Suppl 3), 303-308.

Au, W.W., Bechtold, W.E., Whorton, E.B.Jr. and Legator, M.S. (1995) Chromosome aberrations and response to gamma-ray challenge in lymphocytes of workers exposed to 1,3-butadiene. ***Mutation Research*, 334**, 125-130.

Au, W.W., Wilkinson, G.S., Tyring, S.K., Legator, M.S., Zein, R.E., Hallberg, L. and Heo, M.Y. (1996) Monitoring populations for DNA repair deficiency and for cancer susceptibilty. ***Environ. Health Perspectives,* 104** (Suppl 3), 579-584.

Awa, A.A., Honda, T., Sofuni, T., Neriishi, S., Yoshida, M.C. and Matsui, T. (1971) Chromosome aberration frequency in cultured blood-cells in relation to radiation dose of A-bomb survivors. ***Lancet,* 2**, 903-905.

Azuine, M.A. and Bhide, S.V. (1992) Protective single/ combined treatment with betel leaf and turmeric against methyl (acetoxymethyl) nitrosamine-induced hamster oral carcinogenesis. ***International Journal of Cancer,* 51**, 412-415.

Baker, S.J., Preisinger, A.C., Jessup, J.M., Paraskeva, C., Markowitz, S., Willson, J.K.V., Hamilton, S and Vogelstein, B. (1990) p53 gene mutations occur in combination with 17p alleleic deletions as late events in colorectal tumorigenesis. ***Cancer Research,* 50**, 7717-7722.

Baron, A.E., Franceschi, S., Barra, S., Talamini, R. and LaVecchia, C. (1993) A comparison of the joint effects of alcohol and smoking on the risk of cancer across sites in the upper aerodigestive tract. ***Cancer Epidemiol. Biomarkers,* 2**, 519-523.

Battifora, H. (1994) p53 immunohistochemistry: a word of caution (Editorial). ***Hum. Pathol.* 25**, 435-437.

Bauchinger, M. (1984) Cytogenetic effects in human lymphocytes as a dosimetry system. In: ***Biological Dosimetry*** (Eisert WB, Mendelson, ML, eds). New York: Springer-Verlag, 15-24.

Bender, M.A., Wong, R.M.A. (1982) Biological indicators of radiation quality. In: ***Re-evaluations of Dosimetric Factors Hiroshima and Nagasaki*** (Bond VP and Thiessen JW eds). CONF-810928, Washington: U.S. Department of Energy, 223-240.

Beneditt, E.P. and Benditt, J.M. (1973) Evidence for a monoclonal origin of human atherosclerotic plaques. ***Proceedings of the National Academy of Sciences, USA,* 70**, 1773.

Bennet, W.P., Hollstein, M.C., Metcalf, R.A., Welsh, J.A., He, A., Zhu, S., Kusters, I., Resau, J.G., Trump, B.F., Lane, D.P. and Harris, C.C. (1992) p53 mutation and protein accumulation during multistage human esophageal carcinogenesis. ***Cancer Research,* 52**, 6092-6097.

Bertram, J.S. (2001) The molecular biology of cancer (Review). ***Molecular Aspects of Medicine* 21**, 167-223.

Bhide, S.V., Shivapurkar, N.M., Gothoskar, S.V. and Ranadive, K.J. (1979) Carcinogenicity of betel quid ingredients: feeding mice with aqueous extract and the polyphenolic fraction of betel nut. ***British Journal of Cancer,* 40**, 922-926.

Bhide, S.V., Shivapurkar, N.M. and Gothoskar, S.V. (1984) Arecoline tumorigenicity in Swiss strain mice or normal and vitamin B deficient diet. ***J. Cancer Res. Clin. Oncol.,* 107**, 169-171.

Biaglow, J.E. and Tutle, S.W. (1993) The role of glutathione and associated enzymes in the cellular response to radiation, peroxide and hydroperoxides. ***In Drug Resistance in Oncology,*** Teicher, B.A. (ed) 309-337, Marcel Dekker, New York, 1993.

Blot, W.J., McLaughlin, J.K., Winn, D.M., Austin, D.F., Greenberg, R.S., Preston-Martin, S., Bernstein, L., Schoenberg, J.B., Stemhagen, A. and Fraumeni, J.F.Jr **(1998)** Smoking and drinking in relation to oral and pharyngeal cancer. ***Cancer Research,*** **48**, 3282-3287.

Bonassi, S., Hagmar, L., Strömberg, U., Montagud, A.H., Tinnerberg, H., Forni, A., Heikkilä, P., Wanders, S., Wilhardt, O., Hansteen, I-L., Knudsen, L.E. and Norppa, H. for the European study group on cytogenetic Biomarkers and Health. (2000) Chromosomal aberrations in lymphocytes predict Human cancer independtly of exposure to carcinogens. ***Cancer Research,*** **60**, 1619-1625.

Boveri, T. (1902) Ueber mehrpolige Mitosen als Mittel zur Analyse des Zelkems. Wurzburg, C. Kabitzsch, 1902 and Verch.d, Phys. Med. Ges. Zu Wurzburg, N.F. bd 35 (cited in Boveri, T [1929]. ***The origin of Malignant Tumors***, The Williams and Wilkins Company, P.I.)

Boyland, E. and Nery, R. (1969) Mercapturic acid formation during metabolism of arecoline and arecaidine in the rat. ***Biochemical Journal,*** **113**, 123-130.

Boyum, A. (1968) Separation of leucocyte from blood and bone marrow. ***Scand. J. Clin. Lab. Invest.,*** **21** (S97), 77-89.

Brambilla, E., Gazzeri, S., Moro, D., Caron De Fromentel, C., Gouyer, V., Jacrot, M. and Brambilla, C. (1993) Immunohistochemical study of p53 in human lung carcinomas. ***American Journal of Pathology,*** **143**, 199-210.

Brandt-Rauf, P.W. (1997) Biomarkers of gene expression: growth factors and oncoproteins. ***Environmental Health Perspectives,*** **105**, 807-816.

Brooks, A.L. (1999) Biomarkers of exposure, sensitivity and disease. ***International Journal of Radiation Biology,*** **75**, 1481-1503.

Buckton, K.E., Hamilton, G.E., Paton, L. and Langlands, A.O. (1978) Chromosome aberrations in irradiated ankylosing spondylitis patients. In Evans, H.J. and Lloyd, D.C. (eds.), ***Mutagen–induced chromosome damage in Man,*** Edinburgh University Press, Edinburgh, UK, pp, **142-150**.

Burger, R.M. (1998) Cleavage of nucleic acids by bleomycin. ***Chem. Rev.,*** **98**, 1153-1169.

Cadet, J. (1994) DNA damage caused by oxidation, deamination, ultraviolet radiation and photo exited psoralens, in ***DNA Adducts, Identification and Biological Significance***, Hemminki,K., Dipple,A., Shuker,D.E.G., Kadlubar,F.F., Segerback,D., and bartsch,H., Eds., IARC Sci. Publ., 125, International Agency for Research on Cancer, Lyon, **245**.

Caemano, J., Zhang, S.Y., Rosvold, E.A., Baker, B. and Klein-Szanto, A.J.P. (1993) p53 alterations in human squamous cell carcinoma cell lines. ***American Journal of Pathology,* 142,** 1131-1139.

Caniff, J.P., Harry, W. and Harris, M. (1986) Oral submucous fibrosis its pathogenesis and management. ***Br. Dent. J.,* 160**, 429-434.

Carrano, A.V. and Thompson, L.H. (1982) Sister chromatid exchange and gene mutation. ***Cytogenet. Cell Genet.,* 33**, 57-61.

Castignaro, M. (1988) International N-nitrosamines cheek pouch sample programme: Report on the performance in the 1st study dedicated to determination of nitrosamines in beer and malt. ***Food Additive Contam.,* 5**, 283-288.

Celotti, L., Furlan, D., Ferraro, P. and Levis, A.G. (1989) DNA repair and replication in lymphocytes from smokers exposed in vitro to UV-light. ***Mutagenesis,* 4**, 82-86.

Cerutti, P.A. (1985) Prooxidant states and tumour promotion. ***Science,* 227**, 375-381.

Cesarman, E., Chadburn, A., Inghirami, G., Gaidano, G. and Knowles, D.M (1992) Structural and functional analysis of oncogenes and tumor suppressor genes in adult T-cell leukemia/ Lymphoma shows frequent p53 mutations. ***Blood,* 80**, 3205.

Chagnati, R.S.K., Schonberg, S. and German, J. (1974) A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. ***Proceedings of the National Academy of Sciences,* USA, 71**, 4508.

Chan, G., Boyle, J.O., Yang, E.K., Zhang, F., Sacks, P.G., Shah, J.P., et al (1999) Cyclooxygenase-2 expression is upregulated in squamous cell carcinoma of the head and neck. ***Cancer Research,* 59**, 991-994.

Chang, L.W., Hsia, S.M.T., Chan, P.C. and Hsieh, L.L. (1994) Macromolecular adducts: biomarkers for toxicity and carcinogenesis. ***Anu. Rev. Pharmacol. Toxicol.,* 34**, 41-67.

Charles, K. and Povirk, L.F. (1998) Action of bleomycin on structural mimics of intermediates in DNA Double-strand cleavage. ***Chem. Res. Toxicol.,* 11**, 1580-1585.

Chatterjee, A. and Chattopadhyay, A. (1998) Influence of buthionine sulfoximine mediated glutathione depletion on clastogenic activity of bleomycin and γ-rays. ***Current Science,* 75**, 604-608.

Chatterjee, A. and Deb, S. (1999) Genotoxic effects of arecoline either by the peritoneal or oral route in murine bone marrow cells and the influence of N-acetylcysteine. ***Cancer letters,* 139**, 23-31.

Chatterjee, A. and Jacob-Raman, M. (1986) Modifying effect of reduced glutathione on X-ray induced chromosome aberrations and cell cycle delay in muntjac lymphocytes in vitro. ***Mutation Research,* 175**, 73-82.

Chatterjee, A., Jacob-Raman, M. and Mohapatra, B. (1989) Potentiation of bleomycin induced chromosome aberrations by the radioprotector reduced glutathione. ***Mutation Research,* 214**, 207-213.

Chattopadhyay, A., Choudhary, S. and Chatterjee, A. (1997) Modulation of the clastogenic activity of bleomycin by reduced glutathione, glutathione-ester and buthionine sulfoximine. ***Mutagenesis,* 12**, 221-225.

Chattopadhyay, A., Deb, S. and Chatterjee, A. (1999) Modulation of the clastogenic activity of γ-irradiation in buthionine sulfoximine mediated glutathione depleted mammalian cells. ***International Journal of Radiation Biology, 75***, 1283-1291.

Chiba, I., Shinda, M., Yasuda, M., Yamazaki, Y., Amemiya, A., Sato, Y., Fujinaga, K., Notani, K. and Fukuda, H. (1996) Mutations in the p53 gene and human papillomavirus infection as significant prognostic factors in squamous cell carcinomas of the oral cavity. ***Oncogene,* 12**,1663-1668.

Christensen. J.G., Goldsworthy, T.L and Cattley, R.C. (1999) Dysregulation of apoptosis by c-myc in transgenic hepatocytes and effects of growth factors and nongenotoxic carcinogens. ***Molecular Carcinogenesis, 25***, 273-284.

Ciechanover, A., Digiuseppe, J.A., Bercovich, B., Orian, A., Richter, J.D., Schwartz, A.L. and Brodeur, G.M. (1991) Degradation of nuclear oncoproteins by the ubiquitin system in vitro. ***Proceedings of National Academy of Sciences,*** USA, **88**, 139-143.

Cinciripini, P.M., Hecht, S.S., Henningfield, J.E., Manley, M.W. and Kramer, B.S. (1997) Tobacco addiction; implications for the treatment and cancer prevention. ***Journal of the National Cancer Institute,* 89**, 1852-1967.

Cloos, J., Braekhuis, B.J., Steen, I., Copper, M.P., deVries, N., Nauta, J.J and Snow, G.B. (1994) Increased mutagen sensitivity in head and neck squamous cell carcinoma patients, particularly those with multiple primary tumours. ***International Journal of Cancer,* 56**, 816-819.

Cloos, J., Nieuwenhuis, E.J., Boomsma, D.I., Kuik, D.J., Vandersterre, M.L, Arwert, F., Snow, G.B. and Braekhuis, B.J. (1999) Inherited susceptibility to bleomycin-induced chromatid breaks in cultured peripheral blood lymphocytes. ***Journal of the National Cancer Institute,* 91**, 1125-1130.

Cloos, J., Spitz, M.R., Schantz, S.P., Hsu, T.C., Zhang, Z.F., Tobi, H., Braekhuis, B.J. and Snow, G.B. (1996) Genetic susceptibility to head and neck squamous cell carcinoma. ***Journal of the National Cancer Institute,* 88**, 530-535.

Cohen, S.M. and Ellwein, L.B. (1991) Genetic errors, cell proliferation and carcinogenesis. ***Cancer Research,* 51**, 6493-6495.

Cox, S.C. and Walker, D.M. (1995) *Oral submucous fibrosis: a review.* ***Aust. Dent. J.,* 41**, 294-299.

Dave, B.J., Hsu, T.C., Hong, W. and Pathak, S. (1994) Nonrandom distribution of mutagen-induced chromosome breaks in lymphocytes of patients with different malignancies. ***International Journal of Oncology,*** **5,** 733-740.

Dave, B.J., Trivedi, A.H. and Adhvaryu, S.G. (1991) Cytogenetic studies reveal increased genomic damage among 'pan masala' consumers. ***Mutagenesis,*** **6**,159-163.

Dave, B.J., Trivedi, A.H. and Adhvaryu, S.G. (1992) Role of areca nut consumption in the cause of oral cancers. ***Cancer,*** **70**, 1017-1023.

De Zwart, L.L., Meerman, J.H.N., Commandeur, J.N.M. and Vermeulen, N.P.E. (1999) Biomarkers of free radical damage. Applications in experimental animals and in humans. ***Free Radical Biology and Medicine,*** **26,** 202-226.

Deb, S. and Chatterjee, A. (1998) Influence of buthionine sulfoximine and reduced glutathione on arecoline-induced chromosomal damage and sister chromatid exchange in mouse bone marrow cells in vivo. ***Mutagenesis,*** **13**, 243-248.

Deleve, L.D., and Kaplowitz, N. (1991) Glutathione metabolism and its role in hepatoxicity. ***Pharmacol Ther*** **52**, 287.

Department of health Report (1989) Report on health and social subjects. 35. ***Guidelines for the testing of chemicals for mutagenicity.*** HMSO, London, 100.

Dethlefson, L.A., Lehman, C.M., Biaglow, J.E. and Peck, V.M. (1988) Toxic effects of acute glutathione depletion by buthionine sulfoximine and dimethylfumarate on murine mammary carcinoma cells. ***Radiation Research,*** **114**, 215-224.

Dethmers, J.R. and Meister, A. (1981) Glutathione export by human lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. ***Proceedings of the National Academic Sciences,*** **USA, 78**, 7492-7496.

Dev-Giri, S. and Chatterjee, A. (1998) Modulation of mitomycin C induced sister chromatid exchanges and cell cycle delay by buthionine sulfoximine and reduced glutathione in mouse bone marrow cells in vivo. ***Mutation Research,*** **413**, 227-234.

Di Guilio, R.T., Wasburn, P.C., Wenning, R.J., Winston, G.W. and Jewell, C.S. (1969) Biochemical responses in aquatic animals: a review of determinants of oxidative stress. ***Environ. Toxicol. Chem.,*** **8**, 1103.

Diehl-Jones, W.L. and Boles, N.C. (2000) Use of response biomarkers in milk for assessing exposure to environmental contaminants: The case for dioxin-like compounds. ***J Toxicol. Environ. Health B.,*** **3**, 79-107.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A. Jr., Butel, J.S. and Bradley, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. ***Nature,*** (Lond.) **356**, 215-221.

Dresp, J., Schmid, E. and Bauchinger, M. (1978) The cytogenetic effect of bleomycin on human peripheral lymphocytes in vitro and in vivo. ***Mutation Research,* 56**, 341-353.

Dunham, L.J., Snell, K.C. and Stewart, H.L. (1975) Argyrophilic carcinoids in two syrian hamster (mesocricetus auratus). ***Journal of the National Cancer Institute,* 54**, 507-513.

Edgren, M. and Revesz, L. (1987) Compartmentalised depletion of glutathione in cells treated with buthionine sulfoximine. ***The British Journal of Radiology,* 60**, 723-724.

ENTOX/TIWET (The faculty of the Department of Environmenatl Toxicology and the Institute of Wildlife and Environmental Toxicology In Casarett and Doull's Toxicology. ***The Basic Science of Poisons*** Klaassen CD (ed.) Mc Graw Hill; New York, 1996, 883-905.

Evans, H.J. (1977) Molecular mechanisms in the induction of chromosome aberrations, In Scott, D., Bridges, B.A., Sobels, F.H. (eds.): " ***Programs in Genetic Toxicology"*** Amsterdam: Elsevier/ North Holland, pp **57**.

Fahey, R.C. and Sundqvist, A.R. (1991) Evolution of glutathione metabolism. ***Adv. Enzymol.,* 64**, 1-53.

Field, J.K., Spandidos, D.A., Malliri, A., Gosney, J.R., Yiagnisis, M. and Stell, P.M. (1991) Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of the head and neck tumorigenesis. ***Cancer Research,* 54**, 321-326.

Freedman, J.H., Ciriolo, M.R. and Peisach, J. (1989) The role of glutathione in copper- metabolism and toxicity. ***Journal of Biological Chemistry,* 264**, 5598.

Gardner, P.R. and Fridovich, I. (1993) Effect of glutathione on aconitase in Escherichia Coli. ***Arch. Biochem. Biophys.,* 301**, 98.

Goto, K., Akematsu, T., Shimagu, H. and Suigiyama, T. (1975) Simple differential giemsa staining of sister chromatids after treatment with photosensitive dyes and exposure to light and the mechanism of staining. ***Chromosoma,* 53**, 223-230.

Green, J.A., Vistica, D.T., Young, R.C., Hamilton, T.C., Rogan, A.M. and Ozols, R.F. (1984) Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. ***Cancer Research,* 44**, 5427.

Greenblatt, M.S., Bennet, W.P., Hollstein, M. and Harris, C.C. (1994) Mutations in the p53 tumour suppressor gene: Clues to cancer etiology and molecular pathogenesis. ***Cancer Research,* 54**, 4855-4878.

Greim, H., Csanady, G., Filser, J.G., Kreuzer, P., Schwantz, L., Wolff, T. and Warner, S. (1995) Biomarkers as tools in human health risk assessment. ***Clin. Chem.,* 41**, 1804-1808.

Griffith, O.W. and Meister, A. (1978) Differential inhibition of glutamine and γ-glutamyl cysteine synthetases by α-alkyl analogues of methionine sulfoximine that induce convulsions. ***Journal of Biological Chemistry,* 253**. 2333-2338.

Griffith, O.W. and Meister, A. (1979) Potent and specific inhibition of glutathione synthesis by bithionine sulfoximine (S-n-butyl homocysteine sulfoximine). ***Journal of Biological Chemistry,* 254**, 7558-7560.

Griffith, O.W., Anderson, M.E. and Meister, A. (1979) Inhibition of glutathione biosynthesis by prothionine sulfoximine (S-n- propylhomocysteine sulfoximine), a selective inhibitor of γ-glutamyl cysteine synthetase. ***Journal of Biological Chemistry,* 254**, 1205-1210.

Hagmar, L., Brogger, A., Hansteen, I-L., Heim, S., Hogstedt, B., Knudsen, L., Lambert, B., Linnainmaa, K., Mitelman, F., Nordenson, I., Reuterwall, C., Salomaa, S., Skerfuing, S., Sorsa, M. (1994) Cancer risk in humans predicted by increased levels of chromosomal aberations in lymphocytes: Nordic study group on the health risk of chromosome damage. ***Cancer Research,* 54**, 2919-2922.

Halevy, O., Hall, A. and Oren, M. (1989) Stabilization of the p53 transformation related protein in mouse fibrosarcoma cell lines: effects of protein sequence and intracellular environment. ***Mol. Cell Biol.,* 9**, 3385-3392.

Hall, P.A., McKee, P.H., Menage, H.D., Dover, R. and Lane, D.P (1993) High levels of p53 protein in UV-irradiated normal human skin. ***Oncogene* ,8**, 203-220.

Hall, P.A., Ray, A., Lemoine, N.R., Midgley, C.A., Krausz, T. and Lane, D.P. (1991) p53 immunostaining as a marker of malignant disease in diagnostic cytopathology. ***Lancet,* 338**, 513.

Hammarstrom, S. (1981) Metabolism of leukotrienes C_3 in the guinea pig: Identification of metabolites formed by lung, liver, and kidney. ***Journal of Biological Chemistry,* 256**, 9573.

Harris, C.C. (1996) p53 tumour suppressor gene: from the basic research laboratory to the clinic-an abridged historical perspective. ***Carcinogenesis,* 17**, 1187-1198.

Hay, J., Shahzeidi, S. and Laurent, G. (1991) Mechanisms of bleomycin-induced lung damage. ***Archives of Toxicology,* 65**, 81-94.

Held, K.D., Harrop, H.A. and Michael, B.D. (1982) Reaction kinetics of sulphydryl containing compounds and oxygen with irradiated transforming DNA. ***Radiation Research,* 91**, 304 (abstract).

Hill, L.L., Ouhtit, A., Loughlin, S.M., Kripke, M.L., Ananthaswamy, H.N. and Owen-Schaub, L.B. (1999) Fas ligand: a sensor for DNA damage critical in skin cancer etiology. ***Science,* 285**, 898-900.

Hoffmann, D., Brunnemann, K.D., Prokopczyk, B. and Djordjevic, M.V. (1994) Tobacco-specific H-nitrosamines and areca derived N-nitrosamines: chemistry, biochemistry, carcinogenicity and relevance to humans. ***J. Toxicol Environ. Health,* 41**, 1-52.

Hoffmann, D.J., Heinz, G.H. and Kyrnitsky, A.J. (1989) hepatic glutathione metabolism and lipid peroxidation in response to excess doetary selenomethionine and selenite in mallard ducklings. ***J Toxicol. Environ. Health,*** **27**, 263.

Hoffmann, G.R., Colyer, S.P. and Littlefield, L.G. (1993a) Induction of micronuclei by bleomycin in G_0 human lymphocytes: I. Dose response and distribution. ***Environ. Mol. Mutagen.,*** **21**, 130-135.

Hogson, E. and Levi, P.E. (1994) Introduction to Biochemical Toxicology (2^{nd} edn). ***Appleton and Lange:*** Norwalk, Connecticut.

Hollstein, M.C., Metcalf, R.A., Welsh, J.A., Montesano, R. and Harris, C.C. (1990) Frequent mutation of the p53 gene in human oesophageal cancer. ***Proceedings of National Academy of Sciences, (Wash.),*** **87**, 9958-9961.

Hsu, T.C. (1983) Genetic instability in the human population: a working hypothesis. ***Hereditas,*** **98**, 1-9.

Hsu, T.C., Johnston, D.A., Cherry, L.M., Ramkissoon, D., Schantz, S.P., Jessup, J.M., et al (1989) Sensitivity to genotoxic effects of bleomycin in humans possible relationship to environmental carcinogenesis. ***International Journal of Cancer,*** **43**, 403-409.

Hursting, S.D., Slaga, T.J., Fisher, S.M., DiGiovanni, J. and Phang, J.M. (1999) Mechanism-based cancer prevention approaches: targets, examples and the use of transgenic mice. ***Journal of the National Cancer Institute,*** **91**, 215-225.

Husum, B., Wulf, H.C. and Neibuhr, E. (1986) Sister chromatid exchange frequency correlates with age, sex and cigarette smoking in a 5-year material of 533 healthy adults. ***Hereditas,*** **105**, 17-21.

IARC (1985) IARC monographs on the evaluation of the carcinogenic risks to humans Vol.37. Tobacco habits other than smoking: Betel-quid and areca nut chewing: and some related nitrosamines. ***International Agency for Research on Cancer,*** Lyon, France, 141-200

IARC (1987) Overall evaluation of carcinogenicity: An updating of IARC Monographs; in Monographs of evaluation of carcinogenic risk of chemicals in humans; supplement 7: Volumes 1 to 42, ***International Agency for Research on Cancer,*** Lyon.

Iggo, R., Gatter, K., Bartek, J., Lane, D. and Harris, A.L. (1990) Increased expression of mutant forms of p53 oncogene in primary lung cancer. ***Lancet,*** **335**, 675-679.

Ishii, Y. and Bender, M.A. (1982) Effects of inhibitors of DNA synthesis on spontaneous and ultra-violetlight-induced sister-chromatid exchanges in Chinese hamster cells. ***Mutation Research,*** **79**, 19-32.

Ishikawa, M., Sasaki, K.I. and Takayanagi, Y. (1989a) Injurious effect of buthionine sulfoximine, an inhibitor of glutathione biosynthesis on the lethality and urotoxicity of cyclophosphamide in mice. ***J. Pharmacol. Jap.,*** **51**, 146-149.

Ishikawa, M., Takayanagi, Y. and Sasaki, K. (1989b) I.Modification of cyclophosphamide-induced urotoxicity by buthionine sulfoximine and disulfiram in mice. ***Res. Commun. Path. Pharmacol.,* 65**, 265-268.

Isobe, M., Emanuell, B.S., Givol, D., Oren, M., Croce, C.M. (1986) Localization of gene for human p53 tumor antigen to band 17p13. ***Nature,* 320**, 84-85

Iyawa, K., Tsuda, H., Hiraide, H., Tamaki, K., Tamakura, S., Fukutomi, T., Mukai, K. and Hirahashi, S. (1991) Nuclear p53 immunoreaction asociates with por prognosis of breast cancer. ***Japanese Journal of Cancer Research,* 82**, 835-840.

Jaeschke, H. (1995) Mechanisms of oxidant stress-induced acute tissue injury. ***Proc. Soc. Exp.Biol. Med.,* 209**, 104-111.

Jaslow, R., Offit, K., Lo Coco, F. and Louie, D.C. (1994) Overexpression of p53 in the transformation of follicular lymphoma. ***Lab. Invest.,* 70**, 112A

Jeng, J.H., Chang, M.C. and Hahn, L.J. (2001) Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives (Review). ***Oral Oncology,* 37**, 477-492.

Jeng, J.H., Hahn, L.J., Lin, B.R., Hsieh, C.C., Chan, C.P. and Chang, M.C. (1999) Effects of areca nut, inflorescence piper betel extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. ***Journal of Oral Pathology and Medicine,* 28**, 64-71.

Jeng, J.H., Ho, Y.S., Chan, C.P., Wang, Y.J., Hahn, L.Y., Lei, D., Hsu, C.C and Chang, M.C. (2000) Areca nut upregulates production, cycloxygenase-2 mRNA and protein expression of human oral keratinocytes. ***Carcinogenesis,* 21**, 1365-1370.

Jeng, J.H., Kuo, M., Hahn, L.J. and Kuo, M.Y.P. (1994) Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. ***Journal of Dental Research,* 73**, 1043-1049.

Jeng, J.H., Lan, W.H., Hahn, L.J., Hsieh, C.C. and Kuo, M.Y.P. (1996) Inhibition of the migartion, attachment, spreading, growth and collagen synthesis of human gingival fibroblasts by arecoline, a major areca alkaloid, in vitro. ***Journal of Oral Pathology and Medicine,* 25**, 371-375.

Kandarkar, S.V. and Sirast, S.M. (1977) Changes in vitamin A conditioned hamster cheek pouch epithelium on exposure to commercial shell lime (calcium hydroxide) and tobacco. I. Optical histopathology. ***Journal of Oral Pathology and Medicine,* 6**, 191-202.

Kane, S.A., Natrajan, A. and Hecht, S.M. (1994) On the role of bithiazole moiety in sequence-selective DNA cleavage by Fe^{+} bleomycin. ***Journal of Biological Chemistry,* 269**, 10899-10904.

Kang, Y.J. and Enger, M.D. (1988) Glutathione is involved in the early cadmium cytotoxic response in human lung carcinoma cells. ***Toxicology,* 48**, 93.

Kastan, M.B., Onyckwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. ***Cancer Research,* 51**, 6304-6311.

Kato, S., Bowman, E.D., Harrington, A.M., Blomeke, B. and Shields, P.G. (1995) Human lung carcinogen-DBA adduct levels mediated by genetic polymorphisma in vivo (published erratum appears in J Natl. Cancer Inst., 1995, 87, 861-862) ***Journal of the National Cancer Institute,* 87**, 902-907

Kaur, J., Srivastava, A. and Ralhan, R. (1994) Overexpression of p53 protein in betel and tobacco related human oral dysplasia and squamous cell carcinoma in India. ***International Journal of Cancer,* 58**, 340-345.

Kaur, J., Srivastava, A. and Ralhan, R. (1998) Prognostic significance of p53 protein overexpression in betel- and tobacco-related oral oncogenesis. ***International Journal of Cancer,* 79**, 370-375.

Kehrer, J.P. (1993) Free radicals as mediators of tissue injury and the disease. ***Critical Reviews in Toxicology,* 23**, 21-48.

Kihlman, B.A. (1977) Caffiene and chromosomes. ***Elsevier, Amsterdam, The Netherlands***.

Ko, J.C., Huang, Y.L., Lee, C.H., Chen, M.J., Lin, L.M. and Tsai, C.C. (1995) Betel quid chewing, cigarette smoking and alcohol consumption relayed to oral cancer in Taiwan. ***Journal of Oral Pathology and Medicine,* 24**, 450-453.

Ko, L.J. and Prives, C. (1996) p53, puzzle and paradigm. ***Genes Dev.,* 10**, 1054-1072.

Kosower, N.S. and Kosower, E.M. (1978) The glutathione status of cells. ***Int. Rev. Cytol.,* 54**, 109.

Kuchino, Y., Mori, F., Kasai, H., Inoue, S., Iwai, K., Miura, E. et al. (1987) Misreading of DNA templates containing 8-OH-dG at the modified base at adjacent residues. ***Nature,* 327**, 77-79.

Kuerbitz, S., Plunkett, B., Walsh, W. and Kastan, M. (1992) Wild-type p53 is a cell-cycle checkpoint determinant following irradiation. ***Proceedings of the National Academy of Sciences, USA,* 89**, 7491-7495.

Kwan, H.W. (1976) A statistical study on oral carcinomas in Taiwan with emphasis on the relationship with betel nut chewing: a preliminary report. ***Journal of the Formosan Medical Association,* 75**, 497-505.

Lane, D.P. (1992) p53 guardian of the genome. ***Nature,* 358**, 15-16.

Latt, S.A., Allen, J., Bloom, S.E., Carrano, A., Falke, E., Kram, D., Schneider, E., Schreck, R., Tice, R., Whitefield, B. and Wolff, S. (1981) Sister chromatid exchanges: a report of the gene-Tox Program. ***Mutation Research,* 87**, 17-62.

Lee, F.Y.F., Allalunis-Turner, M.J. and Siemann, D.W. (1987) Depletion of tumour versus normal tissue glutathione by buthionine sulfoximine. ***British Journal of Cancer,* 56**, 33-38.

Lee, F.Y.F., Vesey, A.R. and Siemann, D.W. (1986) Glutathione as a determinant of cellular response to adriamycin. ***NCI monograph*** (see Lee et al, 1987).

Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. ***Cell,* 88**, 323-331.

Li, A.T., Wang, T.D. and Yang, R.T. (1994) Pingyangomycin induced chromosome damage in lymphocytes of laryngeal cancer patients and healthy control subjects. ***Head Neck,* 16**, 510.

Lijinsky, W, and Taylor, H.W. (1976) Carcinogenicity test of two unsaturated derivatives of N-nitropiperidine in Sprague-Dawley rats. ***Journal of the National Cancer Institute,* 57**, 1315-1317.

Liloglou, T., Scholes, A.G.M., Spandidos, D.A., Vaughan, E.D., Jones, A.S. and Field, J.K. (1997) p53 mutations in squamous cell carcinoma of the head and neck predominate in a subgroup of former and present smokers with a low frequency of genetic instability. ***Cancer Research*, 57**, 4070-4074.

Loeb, L.A. (1991) Mutator phenotype may be required for multistage carcinogenesis. ***Cancer Research,* 51**, 3075-3079.

Loeb, L.A. (1994) Microsatellite instability: marker of a mutator phenotype in cancer. ***Cancer Research,* 54**, 5059-5063.

Lu, X. and Lane, D.P. (1993) ***Cell,* 75**, 765-778.

Mac Farlane, G.J., Boyle, P., Evstifeeva, J.V., Robertson, C. and Sculley, C. (1994) Rising trends of oral cancer mortality among mails worldwide: the retum of an old public health problem. ***Cancer Causes Control,* 5**, 259-265.

MacRae, W.D., MacKinnon, E.A. and Stich, H.F. (1979) The fate of UV induced lesion affecting SCEs, chromosome aberrations and survival of CHO cells arrested deprivation of arginine. ***Chromosoma,* 721**, 15-22.

Madden, J.J., Falek, A., Shater, D.A. and Glick, J.H. (1979) Efects of opiates and demographic facrors on DNA repair synthesis in human leukocytes. ***Proceedings of the National Academy of Sciences, USA,* 76**, 5769-5773.

Maher, R., Lee, A.J., Wamakulasuriya, K.A., Lewis, J.A. and Johnson, N.W. (1994) Role of areca nut in the causation of oral submucous fibrosis: a case-control study in Pakistan. ***Journal of Oral Pathology and Medicine,* 23**, 65-69

Maier, H., Dietz, A., Gewelke, U., Heller, W.D. and Weidauer, H. (1992) Tobacco and alcohol and the risk of head and neck cancer. ***Clin. Investig.,* 70**, 320-327.

Majumdar, A.M., Kapadi, A.H. and Pendse, G.S. (1982) Pharmacological properties In: K.V.A. Bavappa (ed.) ***The areca nut palm***, Central Research Institute, 245-261.

Margolin, B.H. and Shelby, M.D. (1985) Sister chromatid exchanges: A reexamination of the evidence for sex and race differences in humans. ***Environ. Mutagen,* 7** (Suppl. 4), 63- 72.

Margolin, B.H., Resnick, M.A., Rimpo, J.Y., Archer, P., Galloway, S.M., Bloom, A.D. and Zeiger, E. (1986) Statistical analysis for in vitro cytogenetic assays using Chinese hamster ovary cells. ***Environ. Mutagen.*, 8**, 183-204.

Marnett, L.J. (2000) Oxyradicals and DNA damage. ***Carcinogenesis,* 21**, 361-370.

Martensson, J.M., Jain, A., Stole, E., Frayer, W., Auld, P.A.M. and Meister, A. (1991) Inhibition of glutathione synthesis in the newborn rat; a model of exogenously produced oxidative stress. . ***Proceedings of the National Academy of Sciences, USA,* 88**, 9360-9364.

Martin, H.M., Filipe, M.I., Mocris, R.W., Lane, D.P. and Silvestro, F. (1992) p53 expression and prognosis in gastric carcinoma. ***International Journal of Cancer,* 50**, 859-862.

Mattison, D.R. (1991) An overview on biological markers in reproductive and developmental toxicology: Concepts, definitions ans use in risk assessment. ***Biomed. Environ. Sci.* 4**, 8-34.

Mayer, J., Warburton, D., Jeffrey, A.M., Pero, R., Walles, S., Andrews, L., Toor, M., Latriano, L., Wazneh, T.D., Tsai, W-Y., Kuroda, M. and Perera, F. (1991) Biologic markers in ehtylene oxide-esposed workers and controls. ***Mutation Research,* 248**, 163-176.

Mc Michael, A. and Hall, J. (1997) The use of biological markers as predictive early-outcome measures in epidemiological research. In: P. Toniolo, P. Boffetta, D.E.G. Shuker, N. Rothman, B. Hulka, and N. Pearce (eds.), ***Application of Biomarkers in Cancer Epidemiology***, pp 281-289. IARC Scientific Publ. No. 142. Lyon, France: IARC.

Meghji, S. and Wamakulasuriya, S. (1997) Oral submucous fibrosis: an expert symposium. ***Oral Disease,* 3**, 276-297.

Meister, A. (1983) Selective modification of glutathione metabolism. ***Science,* 220**, 472-477.

Meister, A. (1988) Modulation of glutathione levels and metabolism. In: P. Cerutti, O.F. Nygaard and M.G. Simic (Eds.), ***Anticarcinogenesis and Radiation Protection***, pp 361-372, New York, Plennum Publishing Corp.

Meister, A. (1991) Glutathione deficiency produced by inhibition of its synthesis and its reversal; application in research and therapy. ***Pharmacol. Ther.,* 51**, 155-194.

Meister, A. and Anderson, M.E. (1983) Glutathione. ***Ann. Rev. Biochem.,* 52**, 711.

Mittelman, F. (1994) Catalog of chromosome aberrations in cancer.***5th Edn., New York, Wiley.***

Modrich, P. (1994) Mismatch repair, genetic stability and cancer. ***Science,* 266**, 1959-1960.

Moldeus, P. and Jiang, Q.G. (1987) Importance of the glutathione cycle in drug metabolism. ***Pharmacol. Ther.,* 33**, 37.

Muir, C.S. and Parkin, D.M. (1985) The world cancer burden: prevent or perish. ***Brit. Med. J.,* 290**, 5-6.

Multani, A.S., Ozen, M., Narayan, S., Kumar, V., Chandra, J., McConkey, D.J., Newman, R.A. and Pathak, S. (2000) Caspase- dependent apoptosis induced by telomere cleavage and TRF 2 loss. ***Neoplasia,* 2**, 339-345.

Nagabhushan, M. and Bhide, S. (1988) Anti-mutagenicity of catechin against environmental mutagens. ***Mutagenesis,* 3**, 293-296.

Naganuma, A., Anderson, M.E., and Meister, A. (1990) Cellular glutathione, is a determinant of sensitivity to mercuric chloride toxicity by giving gluathione monoester. ***Biochem. Pharmacol.,* 40**, 693-697.

Nair, J., Oshima, H., Friesen, M., Croisy, A., Bhide, S.V. and Bartsch, H. (1985) Tobacco specific and betel nut specific N-nitroso compounds: Occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. ***Carcinogenesis,* 6**, 295-303.

Nair, U.J., Floyd, R.A., Nair, J., Bussachini, V., Friesen, M. and Bartsch, H. (1987) Formation of reactive oxygen species and 8-OH-dG in DNA in vitro with betel quid ingredients. ***Chem-Biol. Interact.,* 63**, 157-169.

Nair, U.J., Obe, G., Friesen, M., Goldberg, M.T. and Bartsch, H. (1992) Role of lime in the generation of reactive oxygen species from betel quid ingredients. ***Environmental Health Perspectives,* 98,** 203-205.

Nair, J., Ohshima, H., Pignatelli, B., Friesen, M., Malaveille, C., Calmels, S. and Bartsch, H. (1986) Modifiers of endogenous carcinogen formation: studies on in vivo nitrosation in tobacco users, in: D. Hoffmann, C.C. Harris (Eds.), Banbury Report 23: ***New Aspects of Tobacco carcinogenesis***, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 45-61

Natarajan, A.T., Csukas, I. And VanZeeland, A.A. (1981) Contribution of incorporated 5-bromodeoxyuridine in DNA to the frequencies of sister-chromatid exchanges induced by inhibitors by poly- (ADP-ribose)-polymerase. ***Mutation Research,* 84**, 125-132.

Nery, R. (1971) The metabolic interconversion of arecoline and arecoline-1-oxide in the rat. ***Biochem. J.,* 122**, 503-508.

Oesch, F. and Klein, S. (1992) relevance of environmental alkylating agents to repair O^6-alkylguanine-DNA alkyltransferase: determination of individual and collective repair capacities of O^6-methylguanine. ***Cancer Research,* 52**, 1801-1803.

Ohshima, H., Friesen, M. and Bartsch, H. (1989) Identification in rats of N-nitrosonipectotic acid as a major urinary metabolite of the areca nut alkaloid-derived nitrosamines, N-nitrosoguvacoline and N-nitrosoguvacine. ***Cancer Letters,* 44**, 211-216.

Pandita, T.K. and Hittelman, W.N. (1995) Evidence of a chromatin basis for increased mutagen sensitivity associated with multiple primary malignancies of the head and neck. ***International Journal of Cancer,* 61**, 738-743.

Panigrahi, G.B. and Rao, A.R. (1982) Chromosome breaking ability of arecoline, a major alkaloid, in mouse bone marrow cells in vivo. ***Mutation Research,* 103**, 197-204.

Panigrahi, G.B. and Rao, A.R. (1983) Influence of caffeine on arecoline-induced SCE in mouse bone marrow cells in vivo. ***Mutation Research,* 122**, 347-353.

Panigrahi, G.B. and Rao, A.R. (1986) Study of the genotoxicity of the total aqueous extract of betel nut and its tannin. ***Carcinogenesis,* 7**, 37-39.

Parkin, D.M., Whelan, S.L., Ferlay, J., Raymond, L.A. and Young, J. (1997) Cancer Incidence in five continents Vol. VII. IARC. ***Scientific Publications, no. 143, IARC, Lyon.***

Paul, K., Moitra, P.K., Maity, C.R., Ghosal, S.K. (1996) Teratogenicity of crude areca nut extract in chick embryos. ***Indian Journal of Physiology And Allied Sciences,* 50**, no.4, 182-187.

Perez, R.P., Hamilton, T.C. and Ozole, R.F. (1990) Resistance to alkylating agents and cisplatin; insights from ovarian carcinoma model systems. ***Pharmacol. Ther.,* 48**, 19-27.

Perry, P.E. (1980) Chemical mutagens and sister chromatid exchange, in: F.J. de Seres and A. Hollaender (Eds.), ***Chemical Mutagens: Principles and Methods for Their detection, vol.6.*** Plennum, New York, pp., 1-39.

Pillai, R., Balaram, P. and Reddiar, R.S. (1992) Pathogenesis of oral submucous fibrosis. Relationship to risk factors associated with oral cancer. ***Cancer,* 69**, 2011-2020.

Poirier, M.C. (1997) DNA adducts as exposure biomarkers and indicators of cancer risk. ***Environmental Health Perspectives,* 105**, 907-912.

Povirk, L.F. (1996) DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: bleomycin, neocarzinostatin and other enediynes. ***Mutation Research,* 355**, 71-89.

Povirk, L.F. and Austin, M.J.F. (1991) Genotoxicity of bleomycin. ***Mutation Research,* 257**, 127-143.

Prokopczyk, B., Rivenson, S., Bertinato, P., Brunnemann, K.D. and Hoffmann, D. (1987) 3-(Methylnitrosamino) propionitrile: occurrence in saliva of betel quid chewers, carcinogenicity and DNA methylation in F344 rats. ***Cancer Research,* 47**, 467-471.

Quinlan, D.C., Davidson, A.G., Summers, C.L., Warden, H.E. and Doshi, H.M. (1992) accumulation of p53 protein correlates with a poor prognosis in human lung cancer. ***Cancer Research,* 52**, 4828-4831.

Rabbits, T.H. (1994) Chromosomal translocations in human cancer. ***Nature,* 372**,143-149.

Raghavan, V. and Baruah, H.K. (1958) Areca nut: India's popular masticatory-History, chemistry and utilization. ***Econ. Bot.,* 12**, 315-325.

Rahman, Q., Abidi, P., Afaq, F., Schiffmann, D., Mossman, B.T., Kamp, D.W. and Athar, M. (1999) Glutathione redox system in oxidative lung injury. ***Critical Reviews in Toxicology,* 29**, 543-568.

Rainaldi, R. and Mariani, T. (1982) The distribution of induced sister chromatid exchanges: a tool for identifying agents directly interacting with DNA. ***Mutation Research,* 103**, 333-337.

Ranadive, K.J., Gothoskar, S.V., Rao, A.R., Tezabwalla, B.V. and Ambaye, R.Y. (1976) Experimental studies on betel nut and tobacco carcinogenecity. ***International Journal of Cancer,* 17**, 469-476.

Ranadive, K.J., Ranadive, S.N., Shivapurkar, N.M., and Grothoskar, S.V. (1979) Betel quid chewing and oral cancer: experimental studies on hamsters. ***International Journal of cancer,* 24**, 835-843.

Rao, A.R. (1984) Modifying influences of betel-quid ingredients on B (a) P-induced carcinogenesis in the buccal pouch of hamster. . ***International Journal of cancer,* 33**, 581-586.

Rao, A.R. and Das, P. (1989) Evaluation of the carcinogenicity of different preparations of areca nut in mice. ***International Journal of cancer,* 43**, 728-732.

Reed, D.J. and Fariss, M.W. (1984) Glutathione depletion and susceptibility. ***Pharmacol. Rev.,* 36**, 255.

Revesz, L., Edgren, M. and Wainson, A.A. (1994) Selective toxicity of buthionine sulfoximine to melanoma cell in vitro and in vivo. ***International Journal of Radiation Oncology, Biology Physics,* 29**, 403-406.

Revesz, L., Edgren, M. and Nishidai, T. (1984) Mechanism of inherent radioproctection. ***In. Modification in Cancer Treatment***, Academic Press, Japan, Inc., pp. **13-29**

Rivenson, A., Hoffmann, D., Prokopczyk, B., Amin, S. and Hecht, S. (1988) Induction of lung and exocrine pancreas tumours in F344 rats by tobacco- specific and areca derived N-nitrosamines. ***Cancer Research,* 48**, 6912-6917.

Rosin, M.P. (1984) The influence of pH on the convertogenic activity of plant phenolics. ***Mutation Research,* 1135**, 109-113.

Rothman, N. et al (1995) Incorporating biomarkers into cancer epidemiology: A matrix of biomarker and study design categories. ***Cancer Epidemicol. Biomarkers and Prev.***, **4**, 301-311.

Rupa, D.S. and Eastmond, D.A. (1997) Chromosomal alterations affecting the 1cen-1q12 region in buccal mucosal cells of betel quid chewers detected using multicolor flourescence in situ hybribidization. ***Carcinogenesis,*** **18**, no.12, 2347-2351.

Sagher, D., Karrison, T., Schwartz, J.L., Larson, R.A., Strauss, B. (1989) Heterogeneity of O^6- alkylguanine-DNA alkyltransferase activity in peripheral blodd lymphocytes: relationship between this activity in lymphocytes and in lymphoblastoid lines from normal controls and from patients with Hodgkin's disease or non-Hodgkin's lymphoma. ***Cancer Research,*** **49**, 5339-5344.

Saikia, J.R., Schneeweiss, F.H.A. and Sharan, R.N. (1995) Poly-ADP-ribosylation during induced carcinogenesis in mice in ***Radiation Research,*** (U.Hagen, H.Jeng, C. Streffer ed.) 317.

Sakai, E. and Tsuchida, N. (1992) Most human squamous cell carcinomas in the oral cavity contain mutated p53 tumor-suppressor genes. ***Oncogene,*** **7**, 927-933.

Sander, C.A., Yano, T., Clark, H.M., Harris, C., Longo, D.L., Jaffe, E.S. and Raffeld, M. (1993) p53 mutations is associated with progression in follicular lymphomas. ***Blood,*** **82**, 1994.

Sanghvi, I.D. (1981) Cancer epidemiology: the Indian scene. ***J Cancer Res. Clin. Oncol.,*** **99**, 1-14.

Sarkis, A.S., Dalbagni, G., Cordon-Cardo, C., Zhang, Z.F., Sheinfeld, J., Fair, W.R., Herr, H.W. and Reuter, V.E. (1993) Nuclear overexpression of p53 protein in translational-cell bladder carcinoma: a marker for disease pregression. ***Journal of the National Cancer Institute,*** **85**, 53-59.

Sasaki, M.S. (1982) Sister chromatid exchanges as a reflection of cellular DNA repair. In Sandberg,A.A. (ed.) ***Sister chromatid exchange***, Alan R. Liss, New York, NY, pp 35-164.

Sausville, E.A., Peisach, J. and Horowitz, S.B. (1976) A role of ferrous ion and oxygen in the degradation of DNA by bleomycin. ***Biochem. Biophys. Res. Commun.,*** **73**, 814-822

Sauter, E.R., Ridge, J.A., Gordon, J. and Eisenberg, B.L. (1992) p53 overexpression correlates with increased survival in patients with squamous carcinoma of the tongue base. ***American Journal of Surgery,*** **164**, 651-653.

Schantz, S.P. and Hsu, T.C. (1989) Mutagen-induced chromosome fragility within peripheral blood lymphocytes of head and neck cancer patients. ***Head Neck,*** **11**, 337-342.

Schantz, S.P., Huang, Q., Shah, K., Murty, V.V.V.S., Hsu, T.C., Yu, G., Anderson, P.E., Huvos, A.G. and Raju, S.K. (2000) Mutagen sensitivity and environmental

exposures as contributing causes of chromosome 3p losses in head and neck cancers. ***Carcinogenesis,* 21** (6), 1239-1246.

Schantz, S.P., Spitz, M.R. and Hsu, T.C. (1990) Mutagen sensitivity in patients with head and neck cancers: a biologic marker for risk of multiple primary malignancies. ***Journal of the National Cancer Institute,* 82**, 1773-1775.

Schantz, S.P., Zhang, Z.F., Spitz, M.S., Sun, M. and Hsu, T.C. (1997) Genetic susceptibility to head and neck cancer: interaction between nutrition and mutagen sensitivity. ***Laryngoscope,* 107**, 765-781.

Scott, D., Spreadborough, A., Levine, E. and Roberts, S.A. (1994) Genetic predisposition in breast cancer (letter). ***Lancet,* 344**, 1444.

Scott, N., Sagar, R., Stewart, J., Blair, G.E., Dixon, M.F. and Quirke, P. (1991) p53 in colorectal cancer: clinicopathological correlation and prognosis significance. ***British Journal of Cancer,* 63**, 317-319.

Sen, S., Talukdar, G. and Sharma, A. (1989) A. Betel cytotoxicity. ***J. Ethonopharmacol.,* 26**, 217-247.

Shafer, D.A. (1982) Alternate replication bypass mechanisms for sister chromatid exchange formation. ***Prog. Topics Cytogenet.* 2**, 67-98.

Sharan, R.N. (1996) Association of betel nut with carcinogenesis, ***Cancer Journal,* 9**, 13-19.

Shaw, J.P. and Chou, I.N. (1986) Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts. ***J. Cell Physol.,* 129**. 193-198.

Shin, D.M., Kim, J., Ro, J.Y., Hitelman, J., Roth, J.A., Hong, W.K. and Hittelman, W.N. (1994) Activation of p53 gene expression in premalignant lesions during head and neck tumorigenesis. ***Cancer Research,* 54**, 321-326.

Shirname, L.P., Menon, M.M. and Bhide, S.V. (1984) Mutagenicity of betel quid and its ingredients using mammalian test systems. ***Carcinogenesis,*** (Lond), **5**, 501-503

Shivapurkar, N.M. and Bhide, S.V. (1978) Effect of carcinogenesis (Lond. Betel nut constituents on sulphydryl metabolism. ***Indian J. Phramacol.,* 10**, 257-264.

Shivapurkar. N.M. and Bhide, S.V. (1979) Effect of betel nut constituents on nucleic acid metabolism. ***Indian Journal of Experimental Biology,* 17**, 1141-1144.

Shivapurkar. N.M., Bhide, S.V. and Ranadive, K.J. (1978) Biochemical studies on betel nut constituents. ***Indian J. Pharmacol.,* 10**, 191-200.

Shivapurkar. N.M., Ranadive, S.N., Gothoskar, S.V., Bhide, S.V., Ranadive, K.J. (1980) Tumorigenic effect of aqueous and polyphenolic fractions of betel nut in Swiss strain mice. ***Indian Journal of Experimental Biology,* 18**, 1159-1161.

Shrieve, D.C., Denekamp, J. and Minchinton, A.I. (1985) Effects of glutathione depletion by buthionine sulfoximine on radiosensitization by oxygen and misonidazole in vitro. ***Radiation Research,* 102**, 283-294.

Shugart, L. and Theodorakis, C. (1998) New trends in biological monitoring; Application of biomarkers to genetic ecotoxicology. ***Biotherapy,* 11**, 119-127.

Shugart, L.R. (1996) Biomarkers of DNA damage. ***In Ecotoxicity and Human Health.*** A biological Approach to Environmental Remediation, de Serres FJ, Bloom, AD (eds) Lewis: Boca raton, F123-141.

Sidransky, D. and Hollstein, M. (1996) Clinical implications of the p53 gene. ***Ann. Rev. Med.,* 47**, 285-301.

Silbergeld, E.K. and Davis, D.L. (1994) Role of boimarkers in identifying and understanding environmentally induced disease. ***Clin. Chem.,* 40**, 1363-1367.

Singh, A. and Rao, A.R. (1995) Efect of areca nut,a masticatory, on hepatic drug metabolizing enzymes –SH content and lipid peroxidation in lactating mother and their sucking neonates. ***Cancer Letters,* 92**, 175-180.

Singhal, R.K., Anderson, M.E., and Meister, A. (1987) Glutathione, a first line of defence against cadmium toxicity. ***FASEB Journal,* 1**, 220-233.

Smith, M.L., Chen, I.T., Zhan Q., O'Connor, P.M. and Forance, A.J (1995) Involvement of thep53 tumour suppressor in repair of UV-type damage. ***Oncogene,* 10**, 1053-1059.

Snedecor, G.W. and Cochran, W.G. (1967) ***Statistical methods***, 6th Edn. Iowa State Press, Ames, IA.

Solomon, E., Borrow, J. and Goddard, A. (1991) Chromosome aberrations and cancer. ***Science,* 254**, 1153-1160.

Somers, K.D., Merrick, M.A., Lopez, M.E., Incognito, L.S., Schechter, G.L. and Casey, G. (1992) Frequent p53 mutations in head and neck cancer. ***Cancer Research,* 52**, 5997-6000.

Sorsa, M., Ojajarvi, A. and Salomaa, S. (1990) Cytogenetic surveillance of workers exposed to genotoxic chemicals: preliminary experiences from a prospective cancer study in a cytogenetic cohort. ***Teratogenesis Carcinog. Mutagen.,* 10**, 215-221.

Spitz, M.R., Feuger, J.J., Beddingfield, N.A., Annegers, J.F., Hsu, T.C., Newell, G.R. and Schantz, S.P. (1989) Chromosome sensitivity to bleomycin-induced mutagenesis, an independent risk factor for upper aerodigestive tract cancers. ***Cancer Research,* 49**, 4626-4628.

Spitz, M.R., Feuger, J.J., Halabi, S., Schantz, S.P., Sample, D. and Hsu, T.C. (1993) Mutagen sensitivity in upper aerodigestive tract cancer: a case-control analysis. Cancer Epidemiol. ***Biomarkers Prev.,* 2**, 329-333.

Spitz, M.R., Hoque, A., Trizna, Z., Schantz, S.P., Amos, C.I., King, T.M., et al (1994) Mutagen sensitivity as a risk factor for second malignant tumours following malignancies of the aerodigestive tract. ***Journal of the National Cancer Institute,*** **86**, 1681-1684.

Spivack, S.D., Fasco, M.J., Walker, V.E. and Kaminsky, L.S. (1997) The molecular epidemiology of lung cancer. ***Critical Reviews in Toxicology,*** **27**, 319-365.

Stich, H.F, Bohm, B.A., Chatterjee, K. and Sailo, J. (1983) The role of saliva bome mutagens and carcinogens in the etiology of oral esophageal carcinomas of betel nut and tobacco chewers. In Stich, H.F (ed.), ***Carcinogens and mutagens in the Environment.*** CRC Press, Boca Raton, FL, **vol. 3**, Naturally Occuring compounds: Epidemiology and Distribution, Boca Raton, FL, CRC Press, pp. 43-58.

Stich, H.F. and Anders, F. (1989) The involvement of reactive oxygen species in oral cancers of betel quid/tobacco chewers. ***Mutation Research,*** **214**, 47-61.

Stich, H.F. and Stich, W. (1982) Chromosomal damaging activity of saliva of betel nut and tobacco chewers. ***Cancer Letters,*** **15**, 193-202.

Stich, H.F., Curtis, J.R. and Parida, B.B. (1982) Application of the micronucleus test to exfoliated cells of high cancer risk groups: tobacco chewers. ***International Journal of Cancer,*** **30**, 553-559.

Stich, H.F., Hornby, A.P. and Dunn, B.P. (1985) A pilot beta-carotene intervention trial with Inuita using smokeless tpbacco. ***International Journal of Cancer,*** **36**, 321-327.

Stich, H.F., Rosin, M.P. and Bryson, L. (1982a) Inhibition of mutagenicity of model nitrosation reaction by naturally occurring phenolics, coffee and tea. ***Mutation Research,*** **95**, 119-128.

Stich, H.F., Stich, W. and Parida, B.B. (1982b) Elevated frequency of micronucleated cells in the buccal mucosa of individuals at high risk of oral cancer: betel quid chewers. ***Cancer Letters,*** **17**, 125-134.

Stubbe, J., Kozarich, J.W., Wu, W. and Vanderwall, D.E. (1996) Bleomycins: A structural model for specificity, binding, and double strand cleavage. ***Acc. Chem. Res.,*** **29**, 322-353.

Sundaram, R.K., Bhaskar, A., Vijayalingam, S., Viswanathan, M., Mohan, R. and Shanmugasundaram, K.R. (1996) Antioxidant status and lipid peroxidation in type II diabetes mellitus with and without complications. ***Clin. Sci.,*** **90**, 225-260.

Sundqvist, K., Lui, Y., Nair, J., Bartsch, H., Arvidson, K. and Grafstrom, R.C. (1989) Cytotoxic and genotoxic effects of areca nut related compound in cultured human buccal epithelial cells. ***Cancer Research,*** **49**, 5294-5298.

Suri, K., Goldman, H.M. and Wells, H. (1971) Carcinogenic effect of a dimethyl sulfoxide extract of betel but on the mucosa of the hamster buccal pouch, ***Nature,*** **30**, 383-384.

Suzukake, K., Petro, B.J. and Vistica, D.T. (1982) Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. ***Biochem. Pharmacol.,*** **31**, 121-124.

Suzukake, K., Vistica, B.P. and Vistica, D.T. (1983) Dechlorination of L-phenylalanine mustard by sensitive and resistant tumour cells and its relationship to intracellular glutathione content. ***Biochem. Pharmacol.,*** **32**, 165-167.

Syng-ai, C., Basu Baul, T. and Chatterjee, A. (2002) Antiproliferative and cytotoxic effect of a novel organotin compound on mammalian cells both in vivo and in vitro. ***Mutation Research,*** **513**, 49-59.

Takeshita, M., Grollman, A.P., Ohtusbo, E. and Ohtusbo, H. (1978) Interaction of bleomycin with DNA. ***Proceedings of the National Academy of Sciences,*** (U.S.A.), **75**, 5983-5987.

Tanaka, K., Mori, H., Fujii, M., Takahashi, M. and Hiromo, I. (1983) Carcinogenicity examination of betel quid: II Effect of Vit.A deficiency on rats fed semipurified diet containing betel nut and calcium hydroxide. ***Nutr. Cancer,*** **4**, 260-266.

Tateishi, N., Higashi, T., Naruse, A., Nakashima, K., Shiozaki, H. and Sakamoto, Y. (1977) rat liver glutathione: possible role as a role of cysteine. ***J. Nutr.***,**107**, 51.

Tennant, R.N. (1993) Stratification of rodent carcinogenicity bioassay results to reflect relative human hazard. ***Mutation Research,*** **286**, 111-118.

Tennet, J.E. (1860) Ceylon: An account of the island, physical, historical and topographical...../ with notices of its natural history, antiquities and productions, ***Vol I, 5th rev., London, Longman, Green, Longman and Roberts***, 112-115, 438-439.

Theodorus P.M., Akerboom and Helmut Sies (1981) Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological samples. ***Methods in Enzymology***, **77**, 373-382 (Ed. Jakoby WB).

Thomas, S.J. and Maclennan, R. (1992) Slaked lime and betel nut cancer in Papua New Guinea. ***Lancet,*** **340**, 577-578.

Thor, A.D., Moore, D.H II, Edgerton, S.M., Kawasaki, E.S., Reihsaur, E., Lynch, H.T., Marcus, J.N., Schwartz, L., Chan, L.C., Mayall, B.H. and Smith, H.S. (1992) Acumulation of p53 tumour supressor gene protein: An independent marker of prognosis in breast cancers. ***Journal of the National Cancer Institute,*** **84**, 845-855.

Tice, R.R., Ormiston, B.G. and McFee, A.F. (1989) The effect of agent dose and treatment time on the intracellular distribution of sister chromatid exchanges induced by genotoxic agents in mouse bone marrow cells in vivo. ***Mutation Reasearch,*** **215**, 25-37.

Timbrell, J.A. (1998) Biomarkers in toxicology. ***Toxicology,*** **29**, 1-12.

Tomatis, L., Aitio, A., Wilbourin, J. and Shuker, L. (1989) Human carcinogens so far identified. ***Japanese Journal of Cancer Research,*** **80**, 795-807.

Trivedi, A.H., Roy, S.K. and Patel, R.K. et al (1995) Urine of tobacco/ areca nut chewers causes damage in Chinese hamster ovary cells. ***Carcinogenesis,* 16**, 205-208.

Tucker, J.D., Auletta, A., Cimino, M.C., Dearfield, K.L., Jabobson-Kram, D., Tice, R.R. and Carrano, A.V. (1993b) Sister-chromatid exchange: second report of the Gene-Tox program. ***Mutation Research,* 297**, 101-180.

Vallyathan, V., Green, F., Ducatman, B. and Schulte, P. (1998) Roles of epidemiology. Molecular biology, and biomarkers in the investigation of occupational lung cancer. ***J Toxicol. Environ. Health, B* 1**, 91-116.

Van Wyk, C.W., Oliver, A., De Miranda, C.M., Vander Bij, P. and Grobler-Rabie, A.F. (1994) Observation of the effect of areca nut extracts on oral fibroblast proliferation. ***Journal of Oral Pathology Medicine,* 23**, 145-148.

Van Wyk, C.W., Oliver, A., Hoal-Van Helden, E.G. and Grobler-Rabie, A.F. (1995) Growth of oral and skin fibroblasts from patients with oral submucous fibrosis. ***Journal of Oral Pathology Medicine,* 24**, 349-353.

Vineis, P., Bartsch, H., Capraso, N., Harrington, A.M., Kadlubar, F.F., Landi, M.T et al (1994) Genetically based N-acetyltransferase metabolic polymorphism and low-level environmental exposure to carcinogens. ***Nature,* 369**, 154-156.

Visakorpi, T., Kallioniemi, O.P., Heikkinen, A., Koivula, T. and Isola, J. (1992) Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. ***Journal of the National Cancer Institute,* 84**, 883.

Vogel, E.W. and Nivard, M.J. (1994) International commision for protection against Environmental Mutagens. The subtlety of alkylating agents in reactions with biological macromolecules. ***Mutation Research,* 305**, 13-32.

Walker, C.H., Hopkin, S.P., Sibly, R.M. and Peakall, D.B. (1996) Biomarkers. ***In Principles of Ecotoxicology,*** Walker,C.H., Hopkin,S.P., Sibly,R.M., Peakall,D,B. (eds.) Taylor and Francis: London, 175-194.

Wang, C.K., Su, H.Y. and Lii, C.K. (1999) Chemical composition and Toxicity of Taiwanese Betel Quid Extract. ***Food Chem. Toxicol.,* 37**, 135-144.

Wamakulasuriya, K.A.A. and Johnson, N.W. (1992) Expression of p53 mutant nuclear phosphoprotein in oral lesions. ***Journal of Oral Pathology Medicine,* 21**, 404-408.

Wary, K.K. and Sharan, R.N. (1991) Ctyotoxic and cytostatic effects of arecoline and sodium nitrite on human cells in vitro. ***International Journal of Cancer,* 47**, 396-400.

Wenke, G. and Hoffman, D. (1983) A study of betel quid carcinogenesis I- On the in vitro nitrosation of arecoline. ***Carcinogenesis,*** (Lond), **4**, 169-172.

Wenke, G., Brunnemann, K.D., Hoffmann, D. and Bhide, S.V. (1984) A study of betel quid carcinogenesis. IV. Analysis of the saliva of betel chewers: a preliminary report. ***J. Cancer Res. Clin. Oncol.,*** **108**, 110-113.

Williamson, J.M., Boettcher, B. and Meister, A. (1982) Intracellular cysteine delivering system that protects against toxicity by promoting glutathione synthesis. ***Proceedings of the National Academy of Sciences, USA,*** **79**, 6246-6249.

Wogan, G.N. (1992) Molecular epidemiology in cancer risk assessment and prevention: recent progress and avenues for future research. ***Environmental Health Perspectives,*** **98**,167-178.

Wolff, S., Bodycote, J. and Painter, R.B. (1974) Sister chromatid exchanges induced in Chinese hamster cells by UV irradiation at different stages of the cell cycle: The necessity of cells to pass through S. ***Mutation Research,*** **25**, 73-81.

Wong, D.Y.K., Hsiao, Y.L., Poon, C.K., et al (1994) Glutathione concentration in oral cancer tissues. ***Cancer Letters,*** **81**, 111-116.

Xu, L., Chen, Y.T., Huvos, A.G. et al (1994) Overexpression of p53 protein in squamous cell carcinomas of head without gene mutations. ***Diagn. Mol. Pathol.,*** **3**, 83-92.

Zain, R.B., Gupta, P.C., Warnakulasuriya, S., Shrestha, P., Ikeda, N. and Axell, T. (1997) Oral lesions associated with betel quid and tobacco chewing habits. ***Oral Dis.,*** **3**, 204-205.

Zhan, Q., Bae, I., Kastan, M.B., Fornace, A.J (1994) The p53-dependent gamma ray response of GADD45. ***Cancer Research,*** **54**, 2755-2760.

Appendices:

- Genotoxic effect of raw betel-nut extract and its modulation by buthionine sulfoximine in mammalian cells.
  K. Kumpawat and A. Chatterjee (Communicated)

- A study on raw betel-nut carcinogenesis: Analysis of cytogenetical end-points, p53 expression and endogenous glutathione level of raw betel-nut chewers and their mutagen sensitivity
  K. Kumpawat and A. Chatterjee (Communicated)