Influence of buthionine sulfoximine and reduced glutathione on arecoline-induced chromosomal damage and sister chromatid exchange in mouse bone marrow cells in vivo

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Arecoline (ARC), an alkaloid of the betel nut (Areca catechu), is a major ingredient of betel quid. The carcinogenic potentiality as well as its cell transformation ability has already been reported. Reduced glutathione (GSH), a major non-protein thiol substance plays an important role in protection of cells against the toxic effect of exogenous compounds. In order to understand the role of factors which affect ARC sensitivity, we have made an attempt to establish a relationship between ARC-induced DNA damage and the endogenous GSH status of the cells. ARC was administered to untreated and buthionine sulfoximine (BSO) (a GSH-depleting agent)-treated mice. Exogenous GSH was also added to ARC-administered mice. Cells were fixed at 20 h and both chromosome aberrations (CAs) and sister chromatid exchanges (SCEs) were scored. Both CAs and SCEs were significantly induced by ARC and the frequency of both these parameters were increased further when ARC was given to BSO-treated mice. However, GSH reduced the frequency of CAs induced by ARC but failed to do so for SCEs. The data indicate that ARC-induced DNA damage is influenced by endogenous GSH level. The failure of GSH to reduce the frequency of SCEs indicates that the mechanism of induction of CAs and SCEs by ARC are different.

Introduction

Betel quid chewing is a common habit in oriental countries. Extensive epidemiological studies show that the high incidence of oral and oropharyngeal cancers in these countries is associated with betel quid chewing (Juswala and Deshpande, 1971; Hirayama, 1979; Sanghvi, 1981). During betel quid chewing many carcinogens and mutagens are suspected to be released into the buccal pouch and may play an important role in the process of carcinogenesis (Stich et al., 1983). Arecoline (ARC) (1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester), the major alkaloid of the betel nut, is released into the buccal cavity in the process of chewing the quid. This compound is mutagenic in both bacteria and mammalian cells (Shirname et al., 1983, 1984). It also increases the frequency of micronuclei and chromosomal aberrations (CAs) in bone marrow cells of mice in vivo (Panigrahi and Rao, 1982; Shirname et al., 1984) and in Chinese hamster ovary cells in vitro (Stich et al., 1981). It has also been demonstrated that the frequencies of CAs and sister chromatid exchanges (SCEs) in peripheral blood lymphocytes and micronucleate cells in exfoliated cells of buccal mucosa are increased in areca nut chewers (Dave et al., 1992).

Although ARC has been shown to cause cellular trans-formation in vitro (Ashby et al., 1979), it has not been clearly demonstrated to be carcinogenic in animals (IARC, 1985), although synthetic ARC induces lung tumours in 20% of animals (ICMR, 1978). In vitro experiments report that N-nitrosation of ARC leads to nitroso compounds (Wenke and Hoffmann, 1983) and similar nitrosation is also found in human saliva during chewing of betel quid (Nair et al., 1985; Prokepezyk et al., 1987). Some of these nitroso compounds have cancer inducing ability (Prokepezyk et al., 1987). The interaction of these nitrosamines or their metabolites with cellular targets, particularly DNA, may initiate carcinogenesis.

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 defence against radiation (Howard-Flanders et al., 1963; Held et al., 1982; Chatterjee and Jacob-Raman, 1986) and various toxic effects of xenobiotics (Kosower and Kosower, 1978; Shaw and Chou, 1986) but not against radiomimetic drugs like bleomycin (Chatterjee et al., 1989; Chattopadhyay et al., 1997). The cytotoxicity of ARC could be prevented by addition of extracellular GSH and cysteine, but not by oxygen free radical scavengers like catalase and superoxide dismutase (Jeng et al., 1994). Thus it is of interest to determine the effects of cellular GSH level on the cytotoxicity of ARC. For these reasons much current interest has focused on techniques of reducing cellular levels of GSH prior to treatment with cytotoxic agents. The development of buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, has removed many of the uncertainties due to unwanted side-effects that were associated with GSH depleting agents having less specificity. In the presence of BSO the concentration of GSH in various normal tissues is depleted in a time-dependent manner (Lee et al., 1987) and potentiation of the antitumour activity of anticancer drugs has already been demonstrated (Hamilton et al., 1985; Ono and Shrieve, 1986). Therefore, the present study was undertaken to determine the genotoxic effects of ARC with respect to CAs and SCEs in BSO-treated and untreated mouse bone marrow cells. An attempt has also been made to increase the level of endogenous GSH by adding GSH exogenously and observing its effect on ARC action to provide further information regarding the mechanism of ARC action.

Materials and methods

Male Swiss albino mice, aged 2-3 months and weighing ~25-30 g, maintained in the laboratory in communal cages in a room under controlled temperature (20 ± 2°C), lighting (12 h light/12 h dark) conditions on standard mouse diet (NMC Oil Mills Ltd, Pune, India) and water ad libitum, were used in all experiments. ARC, DL-buthionine-S,R-sulphoximine (BSO), GSH and Hoechst 33258 were purchased from Sigma Chemical Co. (St Louis, MO). 5-bromodeoxyuridine (BrdU) tablets (50 mg) were obtained from Boehringer-Mannheim (Mannheim, Germany). The aqueous drug solutions were freshly prepared before use.
**Treatment of animals and preparation of metaphases**

Arecoline at different concentrations (20, 40 and 60 mg/kg) was injected i.p. 45 min after s.c. implantation of a 50 mg BrdU tablet. BSO (200 mg/kg) was dissolved in phosphate-buffered solution, pH 7.4, and 10 h after BSO treatment ARC was injected. When GSH (400 mg/kg) was used it was injected i.p. 30 min after ARC injection. Marked tremors in the mice resulted after the higher doses of ARC. Cells were fixed 20 h after ARC treatment, preceded by a 2 h colchicine (15 mg/kg) treatment. Animals were killed by cervical dislocation. The femurs were dissected out and bone marrow cells (BMCs) were obtained by injecting 2 ml hypotonic 0.075 M KCl solution (prewarmed at 37°C). Cells were treated in hypotonic solution for 15 min and fixed in 1:3 aceticmethanol. Slides were prepared by the flame drying method.

**Differential staining for sister chromatids**

The method of Goto et al. (1975) was followed. Slides were treated for 10 min with Hoechst 33258 (50 μg/ml) at room temperature in the dark, rinsed in distilled water, mounted in 2% SSC (NaCl, Na citrate, pH 6.8) and kept in sunlight for 30–40 min, depending upon the intensity of sunlight. After rinsing in distilled water the slides were stained in 2% Giemsa (BDH Chemicals Ltd., UK) for 3–4 min, air dried and mounted in DPX.

**Scoring and statistical analysis**

Slides were coded at random. At least 100 well-spread metaphase plates were selected for CA study. CAs were scored as isochromatid breaks (both terminal and interstitial) and chromatid breaks. Exchange aberrations were not found. Translocations were not scored since reciprocal translocation cannot be distinguished without G-banding. Metaphases were categorized as in first, second or subsequent division cycles based on their differential staining patterns. Aberrations were scored from first cycle metaphases (M1) only.

The data were subjected to statistical analysis using the $2 \times 2$ contingency test for frequency of aberrant metaphases. In order to determine the statistical significance of the differences between the ARC, ARC + GSH and BSO + ARC groups for frequency of isochromatid or chromatid breaks a simple $2 \times 2$ test (3×2 tables) was used. The actual observed data were used during statistical analysis. The mean aberration data along with standard error of the mean were analyzed using Microsoft Excel implemented on a Toshiba T2100 computer.

**Results**

ARC-induced CAs and SCEs in mouse bone marrow cells were studied as positive controls to either BSO + ARC- or ARC + GSH-treated mouse bone marrow cells and the results are presented individually in tabular form. Negative control data are also shown. Table I shows that ARC induced a significantly higher frequency of CAs than the negative control. There was not much difference between the 20 and 40 mg/kg ARC treatments, however, with 60 mg/kg induction of aberrations was considerably enhanced. We could not pursue more studies at 60 mg/kg as at this concentration a very high level of tremor was noticed soon after ARC injection and most of the animals failed to survive. The present data indicate that the frequency of CAs induced by ARC increased considerably when ARC was administered to BSO-treated mice. On the other hand, GSH treatment reduced ARC-induced CAs significantly. Induction of exchange aberrations by ARC was not observed in any sample. The effect of BSO itself on formation of spontaneous aberrations was also studied and the data (Table I) showed that it induced significant chromatid breaks with respect to the untreated control. In contrast to BSO, GSH alone failed to induce any aberrations.

Table II shows that BSO increased the frequency of ARC-induced SCEs significantly in second replication cycle cells of mouse bone marrow. The data show that ARC significantly increased the frequency of SCEs with respect to the untreated control, however, the degree of induction was marginally increased at higher ARC concentrations. Interestingly, when GSH was administered the frequency of SCEs induced by ARC was not reduced, rather there was a tendency to increase further. The enhancement of SCE induction by ARC was much higher in BSO-treated than GSH-treated mice. BSO and GSH alone also induced a significantly higher level of SCEs. Table III represents the distribution of SCEs among cells in various treated samples. In ARC-treated samples there was a considerable decrease in the frequency of cells having 0–3 SCEs and a correspondingly significant increase in the frequency of cells having 4–10 SCEs with respect to the untreated control. The dispersion analysis (Table II) indicated that the distribution of SCEs in most of the ARC-treated mice clearly deviated from the Poisson expectation. In BSO + ARC-treated mice the number of metaphases with >7 SCEs was high. The number of SCEs per cell increased sequentially for all cells in ARC + GSH-treated samples, whereas in BSO + ARC-treated samples the number of cells having 0–6 SCEs was dramatically reduced. ARC with or without BSO or GSH showed higher H values, indicating that the SCE values show a considerable spread compared with the untreated control.

**Discussion**

In an effort to understand betel nut-induced carcinogenesis we have studied one of the major alkaloids of the betel nut, namely ARC, with respect to CA and SCE induction, since both parameters are considered to be sensitive indicators of DNA damage which increases the risk of cancer and genetic ill health (Chaganti et al., 1974; Buckton et al., 1978). Our results show that ARC significantly induced both CAs and SCEs in bone marrow cells of mice and this induction could be modified considerably when the mice were treated with either BSO or GSH. Induction of both CAs and SCEs by ARC has already been reported (Panigrahi and Rao, 1982, 1984; Stich et al., 1981). There are also reports regarding induction of SCEs and CAs in peripheral blood lymphocytes of areca nut chusers (Dave et al., 1992). In this study ARC induced mostly chromatid breaks and failed to induce any exchanges. This implies that DNA lesions induced by ARC may not be appearing at the same time or in close proximity, so that they failed to associate to form exchanges (Kihlan, 1977). However, one cannot rule out the possibility that ARC-induced DNA lesions are of a different nature, which are unable to form exchange aberrations since ARC inhibits the level of total poly(ADP) ribosylation of chromosomal proteins, which is important in maintaining chromosomal structure (Saikia et al., 1995).

A novel aspect of the present study is the analysis of the influence of BSO on ARC-induced CAs and SCEs. 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 GSH levels in the various tissues (Griffith and Meister, 1979). It has been demonstrated that following a single dose of 556 mg/kg BSO the GSH concentrations in various tissues were depleted in a time-dependent manner (Lee et al., 1987). Intermediate rates of depletion were seen in the bone marrow, with a nadir at 8–12 h. They observed that the GSH content of bone marrow cells following depletion was 17% of the initial level. Therefore, the incubation period
Table I. Effect of ARC with or without GSH and BSO on induction of CAs in mouse bone marrow cells in vivo

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Total metaphases</th>
<th>Aberrant metaphases (%)</th>
<th>Mean aberrant metaphases</th>
<th>Aberration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Isochromatid break</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>ARC 0</td>
<td>101</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>129</td>
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<td>8</td>
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<tr>
<td>60</td>
<td>107</td>
<td>57</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td>BSO 200</td>
<td>134</td>
<td>9</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>200 + 20</td>
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<td>13</td>
<td>29</td>
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<td>200 + 40</td>
<td>116</td>
<td>32</td>
<td>29*</td>
<td>14b</td>
</tr>
<tr>
<td>GSH 400</td>
<td>112</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ARC+GSH 20</td>
<td>186</td>
<td>3</td>
<td>0</td>
<td>3</td>
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<tr>
<td>400</td>
<td>179</td>
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<td>145</td>
<td>9</td>
<td>7*</td>
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<td>4b</td>
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</table>

*P < 0.001 2×2 contingency x² test.
+bP < 0.001 x² test at df = 2 with respective control values.

for BSO treatment was kept at 10 h in the present study and 200 mg/kg BSO was used. BSO alone induced a significantly higher frequency of CAs and SCEs with respect to the untreated control. However, it was reported earlier that 4 mg/kg BSO could induce SCEs but not CAs in mouse bone marrow cells (Chatterjee et al., 1995). It is most likely that 200 mg/kg BSO could deplete more endogenous GSH than 4 mg/kg, thus inducing CAs besides SCEs. These observations are an indication of the important protective role of endogenous GSH in cells against peroxides and free radicals, which are formed by normal metabolic pathways (Meister, 1983). Thus it appears that BSO-mediated GSH depletion increases the number of DNA strand breaks induced by ARC, thereby enhancing the frequency of both types of aberration. Interestingly, this increased frequency of strand breaks in BSO-treated samples failed to lead to any exchange aberration formation.

In this study we found that exogenous addition of GSH reduced the frequency of CAs induced by ARC but failed to do so for ARC-induced SCEs. However, it has been reported that addition of GSH or cysteine protected oral mucosal cells against ARC cytotoxicity with respect to crystal violet uptake (Jeng et al., 1994). In their study it was observed that the cytotoxicity of ARC could not be prevented by oxygen free radical scavengers like superoxide dismutase and catalase. They suggested that thiol depletion, but not attack by oxygen free radicals, could be the mechanism for ARC cytotoxicity. The present results have demonstrated that BSO-mediated GSH depletion significantly increases the frequency of ARC-induced CAs and SCEs and is therefore in general agreement with Jeng et al. (1994) and also with Sundqvist et al. (1989), who found that betel nut extract and ARC are cytotoxic and can deplete free thiols in human buccal epithelial cells.

The differential influence of GSH on ARC-induced CAs and SCEs is not understood. SCEs are induced efficiently by
substances that form covalent adducts to DNA or interfere with DNA metabolism or repair. Since ARC, by losing only one of its methyl groups during metabolism (Boyland and Nery, 1969), may bind nucleic acids and proteins (Nery, 1971), it may be suggested that such a DNA carrying an adduct may produce SCEs when the cells enter S phase of the cell cycle.
However, one cannot rule out the possibility that a minor fraction of the increased GSH could directly bind to DNA and induce SCEs, since GSH can induce SCEs in mouse bone marrow cells (Chatterjee et al., 1995). It is possible that the initial DNA lesions induced by ARC are processed by the cell to form either a SCE or CA and for the latter case the pathway could be modified by GSH. This implies that the mechanism of SCE and CA formation could be different, as it has already been found that the ratio of induced SCEs to induced mutations or induced CAs is different for each of the chemical mutagens and carcinogens (Carrano et al., 1978; Nishi et al., 1984). Another possibility for the increased frequency of SCEs in ARC+GSH-treated mice could be an additive effect of ARC and GSH, since both could induce SCEs individually. However, the data obtained do not support such an additive effect because ARC and GSH alone induced 6.0 and 5.64 SCEs/cell respectively, while ARC+GSH showed 7.32 instead of 11.64 SCEs/cell. Moreover, ARC is known to react readily with free thiols such as GSH and N-acetylcysteine both in vivo and in vitro (Boyland and Nery, 1969) and this conjugation could be a likely mechanism of thiol depletion and may also render the cells more vulnerable to other reactive agents, such as nitrosamines, which will form from free ARC in the cells within a few hours (Wenke and Hoffmann, 1983). Therefore, the observed enhancement in the frequency of ARC-induced SCEs in BSO-mediated GSH-depleted cells could be attributed to high level production of ARC-DNA adducts and nitrosamines from free ARC, since it was unable to react with endogenous GSH.

There is growing concern regarding the carcinogenic potential of the nitrosation products of ARC (Hoffmann and Hecht, 1985). When ARC is nitrosated in vitro 3-(methylthiynosamino)propionitrile (MNPN), 3-(methylthiynosamino)propionaldehyde (NMPA) and N-nitrosoguvacoline are formed (Wenke and Hoffmann, 1983). Similar nitrosation also occurs in humans during chewing of betel quid and all these nitroso compounds have been detected in the saliva (Nair et al., 1985; Prokepezk et al., 1987). NMPN is a potent and complete carcinogen (Prokepezk et al., 1987). N-Nitrosoguvacoline was also reported to be carcinogenic in an in vivo study in rats (Rivenson et al., 1988). The observed reduction in the frequency of ARC-induced CAs by GSH supports the view that ARC cytotoxicity could be mechanistically linked to thiol depletion. Moreover, the increased frequency of ARC-induced CAs in BSO-mediated GSH-depleted cells further consolidates this view. It seems that exogenously added GSH interacts with most of the ARC and minimizes induction of DNA lesions relevant to CA formation.

The intracellular distribution of SCEs induced in vivo by ARC or BSO+ARC treatment shows that there was an increased number of cells having 7–14 SCEs. In BSO+ARC-treated mice particularly there were very few cells having 0–6 SCEs. Nevertheless, most of the distribution did not deviate from the Poisson expectation. In contrast, non-Poisson distributions were seen in most of the ARC- and ARC+GSH-treated samples. It has been reported that SCEs induced by agents which damage DNA fit well with a Poisson distribution and those from agents that inhibit DNA synthesis are skewed (Rainaldi and Marian, 1982). Therefore, the observed increase in SCEs in BSO+ARC-treated mice could be due to increased DNA damage, while the higher induction of SCEs by ARC and ARC+GSH could be due to inhibition of DNA synthesis. However, both mechanisms of SCE induction are far from being well characterized and the biological basis for this increasing hyperdispersion with increasing mean SCE frequency has not been resolved; it has been proposed that cell-to-cell differences in induced damage, proliferative capacity, cell stage sensitivity and DNA repair activity could modulate the distribution of SCE observed among exposed cell populations (Sasaki, 1982; Shafer, 1982). For the present in vivo study it is considered that specific differences in the pharmacokinetic distribution of reactive species among cycling cells in bone marrow could possibly result in differences in SCE distribution which would not be readily apparent from an analysis of induced SCE frequencies.

Therefore, it may be concluded that the damage induced by ARC in this study could be due to DNA adduct formation (Nery, 1971) as well as the genotoxic potentialities of N-nitroso derivatives and both could be important factors in induction of tumours in betel nut chewers. Because of the close relation between mutagens and carcinogens and extensive human exposure to betel nut, the present study on ARC-induced CAs and SCEs in relation to endogenous GSH level could shed light on the possible carcinogenic effect of ARC through its mutagenic activity. Jeng et al. (1994) suggested that increasing dietary intake of GSH–rich foods or dietary supplementation with GSH may have chemopreventive potential to reduce betel quid or ARC-associated oral lesions, however, the failure of GSH to reduce the level of ARC-induced SCEs in the present study shows a need for a detailed chemopreventive study.

Acknowledgement
This work was supported by a grant from the UGC-NEHU Fellowship, Shillong, India (grant no. G.65-180/Acad95-8143) to S.D.

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Received on June 10, 1997; accepted on December 1, 1997

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