4 EFFECT OF CHLOROQUINE SULPHATE

4.1 Introduction

Malaria remains the world's most important infection in terms of human suffering and death. Over 200 million people have malaria and over 1 million deaths per year are associated with malaria in Africa alone. So, even now, there is a desperate need for practical, effective and safe drugs, insecticides and vaccines to deal with this protozoan infection. Large scale attempts were started in 1950s to eradicate malaria from different parts of the world. Initially the result of the programme was highly satisfactory, the incidence of malaria was greatly reduced in several countries. However, as it stands now, in most tropical areas where the disease is endemic a resurgence is experienced due primarily to development of resistant strains to insecticides and drugs.

The chief agents employed in chemotherapy of malaria are chloroquine (ClQ) and its congeners.

Quinine was earlier used to combat malaria and a number of other types of fevers. As cinchona bark it was introduced into Europe from South America in 1633. Quinine was the principal antimalarial drug until 1930, when based on Ehrlich's work on dyes, mepacrine (Atebrine or Quinacrine), an acridine dye derivative, was introduced. Since then both the drugs were in use. In 1942 when the Japanese army captured South East Asia and the Pacific Islands, the important quinine producing countries,
quinine supply was greatly reduced and a military crisis was precipitated in the Allied forces. Mepacrine was then hastily manufactured to combat malaria among the soldiers. The proper use of mepacrine reduced the malaria rate amongst the Australian troops in New Guinea so astonishingly that daily intake of this drug became a matter of military discipline.

Since then numerous antimalarial drugs have been made and there is a wide choice of remedies. The antimalarial drugs now commonly used are chloroquine sulphate, chloroquine phosphate, amodiaquine, proguanil, mefloquine, primaquine, pyrimethamine, etc. Quinine has lost much of its therapeutic importance because of its numerous side effects. Mepacrine and other acridine derivatives which were largely used are now almost obsolete for malaria; mepacrine is now, however, used as an anthelminthic and an amoebicide.

Chloroquine is one of a large series of 4-aminoquinolines and introduction of this compound as an antimalarial drug is the result of an extensive research on the potentialities of a number of 4-aminoquinolines in the United States during World War II. The aim was to discover more effective and less toxic agents than quinacrine (mepacrine) that has been abandoned as antimalarial drug because of its toxicity and inability to cure vivax malaria. On the basis of the reports of potentials of 4-aminoquinolines as antimalarials made by Russian and subsequently by French investigators a wave of research started in 1943, and hundreds of these compounds were synthesized and tested for their antimalarial effects.
Of these, chloroquine proved most promising. Since then it is in use and is now the most widely used antimalarial drug particularly against *Plasmodium vivax* and *P. malariae* as blood schizontocide and gametocide. It is also used in combination with pyrimethamine (Daraclor) for drug-resistant strains. About 210 metric tons of chloroquine was produced in India alone during the year 1986-87 (OPPI, 1987).

Chloroquine contains the same alkyl side chain as quinacrine, but it differs from the latter in having a quinoline instead of an acridine nucleus and in lacking the methoxy moiety. It has the following structural formula:

![Chloroquine structural formula](attachment://chloroquine.png)

Chloroquine

With regard to potential toxicity at the genetic level of the antimalarial drugs literature records a number of reports. The mutagenic potency of acridine compounds which were earlier used widely as antimalarial agents has been established in microorganisms long back. Acridine compounds like acridine orange (AO), acridine yellow (AY) and proflavine (PF) are known to induce frame-shift mutation in microorganisms through intercalation of
the acridine ring in DNA helix (Calberg-Bacq et al., 1968). They also induce point mutations and gene conversion in yeast (Avers and Dryfuss, 1965) as well as chromosomal aberrations in cultured eukaryotic cells (Ostertag and Kersten, 1965). The Ames' plate reversion and fluctuation test also demonstrated mutagenicity of chloroquine in TA97 (Obaseiki-Ebor and Obasi, 1986) and in TA1537 (Schupbach, 1979) strains of S. typhimurium and in transconjugate strains EE97 and EE102 of E.coli (Obaseiki-Ebor and Obasi, 1986). Quinacrine dihydrochloride was also proved to be mutagenic in S. typhimurium tests strain TA98 (King et al., 1979). In the presence of AO and monochromatic 500 nm light the recombination deficient strain of E. coli WP10 (rec A) showed a 15 fold increase in mutation rate over the wild type (WP2) strain (Hass and Webb, 1979).

Murakami (1973, 1974) studied the colour patterns in F₁ eggs of silkworm one parent of which was treated with acridine compounds like AO, AY, PF, quinacrine hydrochloride (QH) or quinacrine mustard (QM) and reported positive mutagenic actions on the parental chromosomes.

Some DNA intercalating agents like AO, QM and neutral red were found to be highly clastogenic whereas others (quinacrine dihydrochloride and Hoechst 33258) are not in Chinese hamster ovary cells (Hsu et al., 1977). Induction of chromosome breakage by AO in root-tip cells of onion was recorded long ago (Nuti-Ronchi and D'Amato, 1961).

Mutagenic efficiency of acridine compounds and other antimalarial agents have also been assessed in various in vivo and in vitro systems using SCE analysis. Chinese hamster ovary
(CHO) cells exhibited enhanced incidence of SCEs when treated with quinacrine mustard dihydrochloride (Perry and Evans, 1975) and AY, acriflavine (AF) and chloroquine (Raj and Heddle, 1980). Higher incidence of SCEs was also demonstrated in V79 Chinese haster cell line treated with AO and PF (Popescu et al., 1977; Speit and Vogel, 1979); further, the increase was found to be more with exposure to visible light (Speit and Vogel, 1979).

In contrast to the findings in vitro, the intercalating substance like PF failed to induce SCEs in vivo in bone marrow cells of the Chinese hamster (Speit, 1982) and the mouse (Nakanishi and Schneider, 1979). But AO and GM both were shown to elevate the SCE frequency significantly in chick embryos exposed in ovo (Bloom, 1982).

Some complications occurred with acridines and acridine-like compounds while tested using the MNT. Jenssen et al. (1974) claimed induction of MN in bone marrow cells of mice following treatment with frame-shift mutagens. But later on Maier and Schmid (1975) failed to record induction of MN by quinacrine, and noted that fragments recorded as MN by Jenssen et al. (1974) were Feulgen-negative granular or fibrillar inclusions (artifacts). However, production of MN by acridine compounds was recorded later on by some other workers. AF induced MN was reported in bone marrow cells of laboratory mouse (Bruce and Heddle, 1979) as well as in CHO cell line (Heddle et al., 1979). The Chinese hamster ovary cells also exhibited positive effect of AY and chloroquine with MNT (Heddle et al., 1979). Quinacrine hydrochloride was also proved to be an inducer of MN in mouse in vivo system (Bruce and Heddle, 1979).
Sperm head abnormality assay demonstrated positive effect of acriflavine but negative effect of quinacrine dihydrochloride in mouse in vivo system (Bruce and Heddle, 1979).

Though lot of works have been done on the genotoxic potentials of chemotherapeutic agents used against malaria the work on chloroquine, the most widely prescribed antimalarial drug at present, is extremely limited. So far as the author is aware four reports mentioned already - two based on the Ames' *Salmonella* assay system (Schupbach, 1979; Obaseiki-Ebor and Obasi, 1986) and two on cytogenetic analysis on CHO cells (Heddle et al., 1979; Raj and Heddle, 1980), indicate positive effect of the drug. However, we know almost nothing about its potential genotoxicity in vivo. The present study was, therefore, undertaken to evaluate cytogenetic effect of chloroquine sulphate (CQRS) in bone marrow cells of mice.

The protocols followed for chloroquine sulphate are given below:

I. Bone marrow metaphase analysis
   A. Single treatment
      1. Time-response study
      2. Dose-response study

II. Micronucleus test
   A. Dose-response study.

4.2 Materials and Methods

Healthy adult mice of both the sexes weighing about 25 g and of the age group of 10-14 weeks were employed as experimental
animals. Base free sample of chloroquine sulphate (Batch No. GIA-D 3094) was obtained as gift from M/s May and Baker (India) Limited, Bombay. The animals were treated ip with the aqueous solution of the drug. Age and sex matched mice treated ip with water served the purpose of control.

4.2.1 Bone-Marrow Metaphase Analysis

In one set animals were treated with a dose of 25 mg/kg and sampling was done at 4, 8, 16, 32 or 48 h post-treatment; the bone marrow cells of these animals constituted the material for time-response study. In another set some mice received a dose of 50 or 100 mg/kg of the drug and were killed at 8 h post-treatment. The animals of set I killed at 8 h and the animals of set II provided the material for dose-response study. Water injected age-matched mice of either sex were used as controls. 4 animals were employed for each sampling period. Bone marrow preparations, staining of slides and analysis of aberrations were done as described in Materials and Methods (General) (vide 2.1).

4.2.2 Micronucleus Test

Adult mice were treated ip twice with a dose of 25, 50 or 100 mg/kg of the drug at an interval of 24 h and killed at 30 h after the first treatment. Control mice received only water. 5 animals were employed for each dose level. Processing of bone marrow cells for micronuclei preparation, staining of slides and scoring of MN were described in Materials and Methods (General) (vide 2.2).
4.3 Results

4.3.1 Bone Marrow Metaphase Analysis

Qualitative - Qualitatively chromosome aberrations obtained in the control and treated series consisted of gaps, sub-chromatid breaks, chromatid breaks, fragments and unequal chromatids. Gaps were of common occurrence (Fig. 8a). However, no cell was encountered to contain more than four gaps. Chromatid breaks were less common than gaps. In most of the cases the broken acentric fragments were found to lie in the original position without much displacement (Figs. 8b-d). No cell with more than two breaks was noted. Small acentric fragments of untraceable origin were also recorded (Figs. 8e, f). Some polyploid cells were noted in both treated and control series (Fig. 8d). Centromeric separation of the bone marrow chromosomes was not also rare.

Qualitative - Data of control and treated series for different time and dose levels are presented in Table 15 and Table 16 respectively. Since fragments and unequal chromatids resulted due to breakage, they were taken along with the chromatid breaks under the category "break" type aberrations. In no time or dose level statistical analysis of the "break" type aberrations or the total aberrations revealed any significant difference between the control and treated data (Table 15 and Table 16). Students' t-test also revealed the same picture for polyploidy and centromeric separation of the chromosomes.
Explanation for Fig. 8

Photomicrographs of bone marrow metaphases of mice showing various types of structural chromosome aberrations induced by chloroquine sulphate.

a. A chromosome showing a chromatid gap.

b, c. Chromosomes showing chromatid breaks, the fragments without much displacement.

d. A polyploid cell showing a chromatid break in a chromosome.

e, f. Metaphases showing acentric fragments of untraceable origin.
Table 16: Incidence of structural chromosome aberrations in bone marrow metaphases of mice treated once with different doses of chloroquine sulphate. The animals were killed at 8 h post-treatment. Values in parenthesis are mean per 100 metaphases ± S.E.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Metaphases scored/Animals</th>
<th>Break type aberrations</th>
<th>Gaps + Sub-chromat. break</th>
<th>Total aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chromat. break</td>
<td>Frag.</td>
<td>Unequal chromat.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>25</td>
<td>300/4</td>
<td>6</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.67 ± 0.70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(6.00 ± 1.78)</td>
</tr>
<tr>
<td>50</td>
<td>300/4</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.00 ± 0.21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.00 ± 0.21)</td>
</tr>
<tr>
<td>100</td>
<td>300/4</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.00 ± 0.21)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.00 ± 0.21)</td>
</tr>
<tr>
<td>0</td>
<td>(Cont.) 300/4</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.00 ± 0.41)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.00 ± 1.03)</td>
</tr>
</tbody>
</table>

No treated value differs significantly from the control ('t' test).
4.3.2 Micronucleus Test

Qualitative - Micronuclei were recorded both in erythrocytes (PCEs and NCEs) and nucleated cells. In most of the cases the affected cells were with one MN (Figs. 9a-c). However, cells with more than one MN were also available. Micronuclei were as usual small and round (Figs. 9b, d, e). The size of the micronuclei in nucleated cells, however, varied from a small dot to a big mass (Fig. 9f). In certain cases the nucleated cells were found to contain fragmented nuclei (Fig. 9g).

Mitotic metaphases and anaphases were of common occurrence both in control and treated series. Some binucleate cells were scored, where the two nuclei were more or less of equal size (Fig. 9h). Lagging chromosomes (whole or in part) were found in some mitotic anaphases in certain treated individuals (Fig. 9i).

Quantitative - The data on the incidence of micronucleated erythrocytes and nucleated cells are summarized in Table 17. The frequency of erythrocytes (PCEs and NCEs) with MN in the control series was 0.19% which was not markedly different from the value reported by earlier workers in the same species (Chaubey et al., 1975; Das and Swain, 1982; Das and Kar, 1986). In no case in the treated series the frequency of micronucleated cells was significantly higher than the corresponding control value. Further, though polychromatic to normochromatic (PCE/NCE) ratios and proportions of erythrocytes and nucleated cells showed a tendency of decrease, compared to controls, the differences were not highly remarkable.
Explanation for Fig. 9

Cut-out photomicrographs of bone marrow smears showing micronucleated erythrocytes and nucleated cells, binucleate cells and abnormal mitotic figures induced by chloroquine sulphate.

a-e. Poly- and normochromatic erythrocytes, each with one micronucleus.

f. A nucleated cell with one micronucleus.

g. A nucleated cell with fragmented nuclei.

h. A binucleate cell.

i. An anaphase with a lagging chromosome or chromosome fragment.
Table 17: Results of the micronucleus test in bone marrow cells of mice treated with different doses of chloroquine sulphate (ClQS). Animals were treated twice at an interval of 24 hours and killed 6 hours after the 2nd treatment. Values are mean per 100 cells ± S.E. Figures in parentheses are MN/cells scored. For each dose level or control 5 animals were employed.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>PCEs with MN</th>
<th>NCEs with MN</th>
<th>PCEs + NCEs with MN</th>
<th>Nucleated cells with MN</th>
<th>PCE/NCE</th>
<th>RBC/100 Nucleated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.30 ± 0.06</td>
<td>0.12 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>0.09 ± 0.04</td>
<td>0.85 ± 0.06</td>
<td>13.05 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>(15/5, 000)</td>
<td>(6/5, 000)</td>
<td>(21/10, 000)</td>
<td>(8/7, 000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.24 ± 0.04</td>
<td>0.24 ± 0.04</td>
<td>0.04 ± 0.03</td>
<td>0.08 ± 0.02</td>
<td>0.81 ± 0.08</td>
<td>13.05 ± 1.47</td>
</tr>
<tr>
<td></td>
<td>(12/5, 000)</td>
<td>(12/5, 000)</td>
<td>(24/10, 000)</td>
<td>(6/7, 000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.18 ± 0.05</td>
<td>0.20 ± 0.06</td>
<td>0.19 ± 0.04</td>
<td>0.13 ± 0.04</td>
<td>0.83 ± 0.09</td>
<td>14.90 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>(9/5, 000)</td>
<td>(10/5, 000)</td>
<td>(19/10, 000)</td>
<td>(9/7, 000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.22 ± 0.07</td>
<td>0.20 ± 0.06</td>
<td>0.21 ± 0.04</td>
<td>0.17 ± 0.05</td>
<td>1.08 ± 0.09</td>
<td>16.15 ± 1.30</td>
</tr>
<tr>
<td>(Cont.)</td>
<td>(11/5, 000)</td>
<td>(10/5, 000)</td>
<td>(21/10, 000)</td>
<td>(13/7, 000)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No treated value differs significantly from the control ('t' test)
4.4 Discussion

4.4.1 Bone Marrow Metaphase Analysis and Micronucleus Test

Metaphase analysis clearly reveals non-clastogenicity of chloroquine sulphate in mouse *in vivo* system. At no dose or time level chromosome aberrations, including or excluding gap-type aberrations, exceeded the respective control value. The incidence of micronucleated PCEs and NCEs, taking separately or together, at all the dose levels tested remained in the control range. It is indicative of the fact that the drug has neither clastogenic nor spindle poisoning effect. Failure to obtain numerical changes of chromosomes in metaphase analysis following treatment also suggests absence of its effect on the spindle apparatus of the cell. Thus, the results of cytogenetic analyses – metaphase chromosome analysis and MNT, are in very good agreement with each other.

So far as our information goes chloroquine has not earlier been tested for its potential clastogenic effect *in vivo*. However, our finding contradicts those of Heddle et al. (1979) and Raj and Heddle (1980) who have noted higher incidence of MN in Chinese hamster ovary cells treated with chloroquine.

Similarly SCE analysis also reveals positive effect of the drug in CHO cells (Raj and Heddle, 1980). The CHO cells when treated with 5-10 μM of the drug exhibited significant increase in the incidence of SCE as well as micronuclei (Raj and Heddle, 1980). The molecular mechanism leading to the induction of SCE is yet to be known. But the intercalating agents causing frame-shift mutation
seem to behave differently in vivo and in vitro with regard to the induction of SCE. Speit (1982) has demonstrated ineffectiveness of proflavine, methylene blue and chlorpromazine to elevate the SCE frequency over the base-line level in bone marrow cells in vivo of the same species i.e. Chinese hamster. Similarly proflavine was found ineffective in inducing SCEs in bone marrow cells of the mouse (Nakanishi and Schneider, 1979).

Identical situation i.e. effectiveness in vitro and ineffectiveness in vivo, with regard to the induction of MN mentioned above cannot also be ruled out with intercalating agents. Besides, there is a question of species specificity; our work is based on the mouse. Chloroquine like acridine compounds, is known to bind to microbial as well as mammalian DNA (Rollo, 1975; Michael and Williams, 1974). Earlier Maier and Schmid (1975) on the basis of their work with quinacrine, an acridine compound, put forward the view that the intercalating agents cannot induce MN in bone marrow cells of the mouse.

Chloroquine is rapidly and almost completely absorbed from the gastrointestinal tract and less than 10% of the administered dose is passed out with the stool. It undergoes appreciable biotransformation (see Webster, 1985). The metabolites are desethylchloroquine, bisdesethylchloroquine, and some uncharacterized metabolites. The first metabolite constitutes about one fourth of the total material appearing in the urine. Slightly more than half of the urinary drug-products can be accounted for as unchanged chloroquine. It is deposited in the tissue in considerable amount. After single or weekly doses, the half-life of the drug in plasma is about 3 days. The negative
effect obtained by us can, therefore, not be attributed to the fast elimination or biotransformation of the drug. As our study covers 64 hours post-treatment period it can be argued that the metabolites of the drug mentioned above are also ineffective in causing chromosome aberration.

Considerable evidence has accumulated suggesting that CLQ forms an intercalated complex with DNA (Cohen and Yielding, 1965; Waring, 1970). Though there is no correlation between intercalating ability and mutagenicity (Drake, 1970) CLQ has been proved to be mutagenic in bacterial systems (Schupbach, 1979; Obaseiki-Ebor and Obasi, 1986). Its clastogenic efficiency has also been discussed in mammalian cells in culture (Heddle et al., 1979; Raj and Heddle, 1980). However, it fails to induce any chromosome damage in mammalian system in vivo. It seems that binding of CLQ to DNA is not sufficient to cause chromosome breakage.

The human therapeutic dose of chloroquine varies greatly on the basis of severity of infection and age. The normal adult dose is 500 mg per day; in case of acute attack as high as a dose of 1.5 g is administered. For a man of 50 kg body weight the normal adult dose comes to 10 mg/kg. So, the highest dose employed in the present study (100 mg/kg) which is on per kg basis equivalent to 10 times the normal human therapeutic dose even fails to demonstrate any clastogenic effect. However, here, instead of repeated treatment for a few weeks, which is the usual mode of therapy, the drug was administered only once in case of metaphase analysis and twice in case of MNT.