Chapter - 7

Anticlastogenic Activity of Brahma Rasayana
7.1 Introduction

Cancer is the result of the accumulation of multiple genetic changes that occur in a single clone of cells. Each alteration, irrespective of a initiation or a progression associated event may be mediated through a gross chromosomal damage. Radiation induced reciprocal translocations such as have occurred in the cell may be passed on through many generations of cell replication and emerge in clonal populations (Kano and Little, 1984; 1985). Such deletions and translocations can result in gene mutations, Mutation is the sudden heritable changes formed as a result of genetic changes such as chromosomal aberrations, micronuclei (Nagalaskshmi et al, 1995) and sister chromatid exchanges (Perera et al, 1992) Cancer cells are aneuploid and contain multiple stable chromosomal aberrations. The major potential consequence of radiations induced mutations in animal systems (National Research, Council, 1990) is heritable genetic effects resulting from mutations induced in germinal cells.

The free radical products of water radiolysis which are supposed to be primarily responsible for the clastogenic effects of radiation. The products are superoxide anion radicals (O$_2^\cdot$) and hydrogen peroxide (H$_2$O$_2$). Antioxidant enzymes such as superoxide dismutase converts O$_2^\cdot$ to O$_2$ and catalase decomposes hydrogen peroxide. Brahma Rasayana (BR) is a polyherbal drug preparations which has high antioxidant property in vitro and in vitro (Chapter II) and also has increased the level of endogenous antioxidant system in irradiated mice. Based on these properties, the present chapter examines the effect of BR on clastogenic changes such as chromosomal aberrations and micronuclei formation in the bone marrow of irradiated mice.

7.2. Material and Methods

May-Grunwald stain solution (0.25% in methanol) was obtained from Loba Chemie PVT. Ltd, Mumbai, Giemsa stain was obtained from E -Merck (India) Pvt. Ltd Mumbai. Foetal calf serum (FCS) was obtained from Biological industries, Kibbutz Beit Haemek, Israel, Colchicine was obtained from Sisco
7.2.a. Animals

Inbred strains of Swiss albino mice (4-5 weeks old, 20-25g) were purchased from our animal house and were maintained at air and temperature controlled rooms provided with normal mouse chow (Saifeeds, Bangalore, India) and water ad libitum.

7.2.b. Radiation treatment

Whole body radiation was given using cobalt -60 teletherapy unit (Theratron 780, Canada). Animals were kept in specially constructed restraining boxes with a capacity of holding ten mice and irradiated by Gamma rays (100 rads/min.).

7.2.1. Effects of BR on radiation induced micronuclei formation

Micronucleus in a cytogenetic screening procedure for the detection of freshly induced structural chromosome aberrations. This test is based on the principle that chromatin fragment which may be produced by the clastogenic agents or spindle poisons, lag behind during anaphase due to the detachment from the centromeric portion of the chromosome. In anaphase this acentric chromatid or chromosome fragments or entire chromosome is left behind, when chromosome move towards spindle poles. After telophase, regular daughter nuclei are formed. The lagging elements of chromosome are included in the daughter cells, but fail to incorporate with main nucleus and are transformed into one or several secondary nuclei. These are much smaller and referred to use micronuclei.

Inbred strains of swiss albino mice (4-5 weeks old) weighing 20-25g were used for this experiment. They were divided into five groups of 4 animals each. Group I served as normal. Group II kept as irradiated control. Group III, IV & V were treated with five daily doses of BR (10, 50 and 100mg/does./mouse, po respectively). All control and treated animals were received single exposure of whole body radiation (150 rads/mouse) before last dose of drug administration. 24hr after radiation treatment all animals were sacrificed by cervical dislocation.
and bone marrow from both femurs was suspended in foetal calf serum (FCS) and
smears were made. Four slides were prepared from each animal (Schmid, 1975).
The slides were air dried, fixed in absolute methanol for 5 min. and stained with
0.25% May Grunwald (MGG) stain for 3 min. again treated with diluted MGG
(One part of stain with one part of distilled water) for 2 min. The slides were again
stained with Giemsa (One part of stain with six parts of distilled water) for 10 min.
followed by washing in distilled water, air dried and observed under microscope.
The incidence of micronuclei (MN) in polychromatic erythrocytes (PCE), and the
ratio of polychromatic to normochromatic erythrocytes were scored at 100x under
oil immersion. The polychromatic/normochromatic erythrocyte ratio was based
on a minimum of 2000 erythrocytes. From each animal 2000 polychromatic
erythrocytes (PCE) and corresponding normochromatic erythrocytes (NCE) were
scored for the presence of micronuclei (MN).

7.2.2. Effect of BR on radiation induced chromosomal aberrations (Bone marrow metaphase Analysis)

This analysis detects the agents which are carcinogenic or mutagenic
capable of inducing structural changes in the chromosome. Chromosomal
aberrations are microscopically visible changes in chromosome structure. They
may be complete breaks of single chromatids resulting in a loss or deletion of part
of the chromosome material. The deleted material appears as fragments in
metaphase preparations. The broken chromatids may rejoin to form simple or
complex configurations Discontinuities within chromatid arms, in which the
chromatid region distal to the discontinuity is aligned with the rest of the
chromatids are called gaps or achromatic lesions. If the unstained region is greater
than the diameter of the chromatid, and provided there is no evidence of the
linking strands of material across the discontinuity, they are called as breaks.
These chromosomal aberrations occur because of the lesions in the DNA that lead
to discontinuities in the DNA double helix. The primary lesions include single and
double strand breaks, base damage, DNA-DNA and DNA protein cross links,
alkylation at nucleic acid base or phosphate groups. Intercalations, thymine
dimers, a purine and a pyrimidine sites are recognized by DNA repair process.
Therefore the lesions may be corrected or transformed to restitute the original base sequence or produce chromosomal aberrations and or gene mutations.

Inbred strains of male swiss albino mice (4-5 weeks old) weighing 20-25 gm were used for this study. Animals were divided into five groups of four animals each. Group I served as normal. Group II kept as irradiated control. Group III, IV and V were treated with five doses of BR (10; 50 and 100 mg/dose/mouse, po respectively). Both control and treated animals received single exposure of whole body radiation (300 rads/mouse) before last dose of drug administration. Colchicine (20μg/mouse, ip) was injected 90 min. before the animals were killed. 24 hour after irradiation, animals were killed by cervical dislocation and bone marrow were analysed for chromosome abnormalities (Alder, 1988; Nicolar et al., 1971). According to this method, bone marrow was collected from both femurs of each mice, flushed out into a test tube containing 5 mL of isotonic saline. The cell pellets were centrifuged for 5 min at 800 rpm. The supernatant was discarded, cells were treated with hypotonic solution (preincubated 0.075 M KCl at 37°C) for 25 min. at 37°C and centrifuged for 5 min. at 800 rpm. The cell pellet was fixed with freshly prepared fixative (methanol: glacial acetic acid, 3:1) by adding drop by drop keeping the centrifuge tubes on the cyclomixer, made up to 3 mL volume and tubes were left at 4°C for 20 min. Cells were washed with the fixative thrice before slide preparation. A suspension of fixed cells is dropped on to chilled microscopic slide, held inclinally in such a way that the nuclei burst and the chromosomes are released. The chromosomes become firmly attached to the slides as the fixative dried over the slide warmer. Four slides were prepared from each animal and stained with Giemsa (Giemsa solution was prepared by mixing 2 mL of Giemsa stock solution with 2 mL of phosphate buffer and making up to 50 mL with distilled water in a coplin jar). Slides were immersed in the stain for 10 min., washed with distilled water and air dried slides were scanned for metaphase plates under low power and the chromosomes were examined under oil immersion. A minimum of 100 metaphase plates were scored for aberrations.
7.3. Statistical Analysis

Data was expressed as mean ± standard deviation. Significance levels for comparison of differences were determined using student's t test.

7.4. Results

7.4.1. Effect of BR on radiation induced micronuclei formation

Radiation dose of 150 rads was found to affect the multiplication leading to the formation of young erythrocytes of polychromatic erythrocytes (Table 17). In irradiated control mice, the frequency of micronucleated polychromatic erythrocytes (% MnPCE) were 3.11±0.46 and micronucleated normochromatic erythrocytes (%Mn NCE) were 0.94±0.07 when compared to normal mice, where %Mn PCE and %Mn NCE were 0.33±0.02 and 0.26±0.04 respectively. Oral administration of BR (10, 50 and 100mg respectively) in irradiated mice were found to be significantly (p<0.001) reduced the frequency of %Mn PCE (0.95±0.14, 0.67±0.3 and 0.77±0.07 respectively) and % MnNCE (0.57±0.02, 0.59±0.02 and 0.64±0.01 respectively) The percentage of polychromatic erythrocytes (% PCE) were significantly increased in BR treated irradiated mice (10, 50 and 100mg respectively) and the values were found to be 37.5±0.76, 42.2±0.82 and 40.7±0.68 respectively, when compared to irradiated control mice (% PCE=26.4±0.6). There was a significant increase in the ratio of polychromatic to normochromatic erythrocytes (PCE/NCEx100) in BR treated (10,50 and 100mg respectively) irradiated mice and the values were found to be 60.1±1.19, 73.7±2.41 and 68.7±1.88 respectively when compared to irradiated control mice (PCE/NCEx100=35.9±1.19) These parameters indicates that BR has significant anticlastogenicity.

7.4.2. Effect of BR on radiation induced chromosomal aberrations

In normal mice the number of chromosomal fragment per 100 metaphase cells were 22.5±6.42 and percent of abnormal cells were only 8.8±1.09 (Table 18). Irradiation was found to be increased the number of fragments per 100
Table - 17

Effect of Brahma Rasayana (BR) on radiation induced micronuclei formation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%Mn PCE</th>
<th>% MnNCE</th>
<th>%(MnPCE + MnNCE)</th>
<th>% PCE</th>
<th>PCE/NCE X 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.33±0.02</td>
<td>0.26±0.04</td>
<td>0.29±0.02</td>
<td>47±0.7</td>
<td>88.9±2.24</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>3.11±0.46</td>
<td>0.94±0.07</td>
<td>1.54±0.18</td>
<td>26.4±0.6</td>
<td>35.9±1.19</td>
</tr>
<tr>
<td>Radiation + BR (10mg)</td>
<td>*0.95±0.14</td>
<td>*0.57±0.02</td>
<td>*0.71±0.05</td>
<td>*37.5±0.76</td>
<td>60.1±1.19</td>
</tr>
<tr>
<td>Radiation + BR (50mg)</td>
<td>*0.67±0.03</td>
<td>*0.59±0.02</td>
<td>*0.62±0.02</td>
<td>*42.2±0.82</td>
<td>73.7±2.41</td>
</tr>
<tr>
<td>Radiation + BR (100mg)</td>
<td>*0.77±0.07</td>
<td>*0.64±0.01</td>
<td>*0.69±0.03</td>
<td>*40.7±0.68</td>
<td>68.7±1.88</td>
</tr>
</tbody>
</table>

Values are mean ±SD of 4 animals
*p<0.001
metaphase cells (387.5±54.5) by increasing the number of gaps (123.3) chromosome breaks (162.3), Chromatid breaks (51.8) and exchanges (50.3) and also increased the percent of abnormal cells (81.5±5.72). Oral administration of BR (10,50 and 100mg respectively) significantly decreased the number of fragments per 100 metaphase cells (200.8±21.09, 89.5±13.65 and 106.3±25.53 respectively) and percent of abnormal cells were 62.5±5.32, 32±1.58 and 34.5±5.68 respectively when compared to irradiated control mice. This study also indicates that BR can ameliorate the radiation induced clasogenic changes.

7.5. Discussion

The mutagenic effects of ionizing radiation were first described by Herman Muller in 1927 in his classic experiments with the fruit fly Drosophila. Mechanism of action of radiation on DNA is two types, one is direct action and other is indirect action of ionizing radiation and indirect action is by the production of highly reactive water-derived free radicals. DNA structural analyses have shown that the majority of radiation induced mutations in human cells (Li et al., 1992) result from large scale genetic events involving loss of the entire active gene and often extending to other loci on the same chromosome.

Radioprotectors can protect the tissues from the undesirable side effects of radiation. The radioprotective effectiveness of free-radical scavengers largely correlates with their ability to scavenge hydroxyl radicals produced by the interaction of gamma rays in the vicinity of cellular targets. In the previous chapters we have clearly revealed that BR could inhibit the oxygen radicals such as superoxides, hydroxyl radicals lipid peroxidation in vitro, and could inhibit both superoxide radicals and nitrite ions (in vitro and in vivo). Moreover BR could enhance the endogenous antioxidant systems in normal and irradiated mice.

Exposure to ionizing radiation was found to affect the multiplication of bone marrow can lead to the formation of young poly chromatic erythrocytes, increase in micronuclei formation and increase the number of chromosome breaks, chromatid breaks, gaps and exchanges per 100 metaphase stage cells of
Table 18

Effect of Brahma Rasayana (BR) on radiation induced chromosomal aberrations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in metaphase cells</th>
<th>No. of fragments /100 metaphase cells</th>
<th>% abnormal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gaps</td>
<td>Chromosome breates</td>
<td>Chromatid breaks</td>
</tr>
<tr>
<td>Normal</td>
<td>3.5</td>
<td>4</td>
<td>15.3</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>123.3</td>
<td>162.3</td>
<td>51.8</td>
</tr>
<tr>
<td>Radiation + BR</td>
<td>31.3</td>
<td>88.3</td>
<td>60</td>
</tr>
<tr>
<td>(10mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiation + BR</td>
<td>16.3</td>
<td>42.3</td>
<td>25.8</td>
</tr>
<tr>
<td>(50mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiation + BR</td>
<td>27.5</td>
<td>43.8</td>
<td>21</td>
</tr>
<tr>
<td>(100mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ±SD of 4 animals

*p<0.005, **p<0.001
Figure 46

Effect of Brahma Rasayana (BR) on Micronuclei Formation and Chromosomal Aberrations in Irradiated Mice

a) Polychromatic erythrocytes with micronuclei

b) Normal metaphase chromosome

c) Aberrated metaphase chromosome
each animal. Oral administration of BR was found to be significantly reduce the above parameters indicating BR is a potential anticlastogenic agent. BR was found to reduce the myclosuppression in cancer patients receiving chemotherapy and radiation (Joseph et al., 1999). One of the main ingredient in BR, *Empica officinalis* has high antioxidant potency (Jose and Kuttan, 1995). BR is not only useful as an immunomodulatory agent but also to reduce the harmful effects of radiation mediated free radicals and thus prevent the disease.