3. MATERIALS AND METHODS (GENERAL)
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3.1 Sample Selection: Patients referred to the Division of Human Genetics, St. John’s Medical College, Bangalore, India, by various hospitals in and around Bangalore were enrolled for this study. Such patients were initially diagnosed by the clinicians at the Division and only those patients who confirmed to a disease condition criteria were selected for this study.

The diseases studied were as follows:

1. Ataxia telangiectasia (A-T)
2. Aplastic anaemia (AA)
3. Polyposis coli (PC)
4. Down syndrome (DS)
5. Klinefelter syndrome (KS)
6. Marfan syndrome (MS)
7. Turner syndrome (TS)

The sample size and the type of diseases investigated differed for different experiments. These have been mentioned in the 'Material and Methods' for each chapter.

As far as possible, blood from the patients and controls matched for age and sex were X-irradiated at the same time but in different Petri-dishes.
Controls as well as patients selected for this study did not have any of the vitiating factors mentioned below:

(a) bacterial and viral diseases for the past three years,
(b) smoking or chewing tobacco in any form
(c) alcoholism
(d) exposure to radiation (diagnostic or therapeutic doses)
(e) exposure to antibiotics for the past three months

3.2 X-ray Equipment, Irradiation Condition and Calibration: 100 KVp superficial X-ray machine (Maximer) was used for this study. It was operated with a tube current of 5mA at a dose rate of 70 rads/minute. An Aluminium filter of 1 m.m. thickness and a metal cone of 30 cms. height and 15 cms. diameter (distal end) was fitted to the X-ray equipment. A Wax Block of (5 cms.) thickness was placed below the source for better dispersion of X-rays. The Blood sample stored in a plastic Petri-dish was placed on the wax block. The cone along with the X-ray equipment was pulled down and locked, so that the Petri-dish was at the centre of the cone. Irradiation was carried out at room temperature (26 - 30°C).
The X-ray unit was calibrated by the physicists of the Radiation Physics Unit of Kidwai Memorial Institute of Oncology, Bangalore at regular intervals using the 'Victoreen Ionizing Chamber'.

3.3 LABORATORY METHODOLOGY

Sterilization of Glass Ware: All glass ware and filtration apparatus were dipped in a solution of Teepol (BDH) ie: 15 ml Teepol in 1 litre of water, overnight (17-24 hrs.). It was cleaned using a brush and washed thoroughly in running tap water, rinsed several times in glass-distilled water and boiled for one hour. They were then dried in an hot-air oven at 120°C for about one hour. After cooling they were packed with aluminium foil and brown paper and autoclaved at a pressure of 15 pounds / square inch for 20 minutes. They were then dried in an hot-air oven, cooled and stored in a sterile chamber.

Preparation of Culture Medium and Serum: RPMI 1640 (GIBCO) media was prepared in sterile distilled water and filtered using positive-pressure-pump (Millipore) with 0.22 micron (pore size) cellulose filter. Following this Antibiotic-solution (GIBCO) containing penicillin and streptomycin was added and stored at 4°C.
Clotted human blood (AB+) was obtained from the blood bank after routine tests and the bottle was kept tilted overnight till the cells had settled down. The serum samples were centrifuged at 1000 rpm twice, after discarding the cell pellets. These samples were then heat inactivated at 56°C for 45 minutes in a water bath (Memmert) and filtered using positive-pressure filtration system with 0.45 micron (pore size) cellulose filter (Millipore). The samples were aliquoted into 20 ml vials and frozen.

Blood Collection: About 20 ml of venous blood was collected under sterile condition using a anticoagulant heparin 1000 IU/ml concentration. The blood was aliquoted into 0.6ml to four inches diameter plastic Petri-dishes for irradiation. Within an hour the Petri-dishes were transported to Kidwai Memorial Institute of Oncology, Bangalore, for irradiation. Duplicate cultures were set up for each dose.

LYMPHOCYTE CULTURE: (Hungerford et al.,1965)
Filtered RPMI 1640 media containing antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) was supplemented with 20% serum and 30 μl/ml PHA-M (GIBCO) and 5 μg/ml of 5-Bromo 2’ deoxyuridine (BrdU; SIGMA). For each dose, duplicate cultures were set up in 15 ml
sterile vials (Borosil) using 5 ml culture media and 0.03 ml of irradiated blood. The cultures were incubated at 37°C for 48 and 72. The cultures were exposed to 0.02 μg/ml Colcemid (GIBCO) for the last two and half hours before harvesting.

HARVESTING: At the end of 48 or 72 hours the culture vials were gently shaken and transferred to 15 ml centrifuge tubes (Borosil) and centrifuged at 10000 rpm for 8 minutes. The supernatant was discarded and the cells mixed well and hypotonic treatment was given with 5 ml of 0.075 M potassium chloride solution (prewarmed at 37°C) added and mixed well. The cells were incubated at 37°C for 15 minutes and at the end of this period the cells were centrifuged and the supernatant was discarded without disturbing the leucocyte-layer. The cells were mixed well and the freshly prepared fixative (methanol and glacial acetic acid mixed in the proportion of 3:1) was added drop-by-drop keeping the centrifuge tubes on the cyclomixer. Five ml of fixative was added and the centrifuge tubes was left in the refrigerator at 4°C for 20 minutes. After this 3 changes of fixative were made and each time centrifugation was at 1000 RPM for five minutes. After the last change about 0.3 to 0.5 ml of fixative was added and mixed. Two or three drops of cell
suspension was dropped onto a chilled slide from a distance of about 60 cms. Before slide preparation, the glass slides were individually washed with soap, cleaned in running tap-water and rinsed in glass-distilled water. They were then rinsed in 90% ethanol and stored at 4°C for at least 2 hours. The slides were heat dried and stored for staining. At least 4 to 5 slides were prepared for each experiment.

STAINING

GIEMSA STAINING: The slides were stained for 5 minutes in 5% Giemsa solution (BDH) in deionized water. The slides were rinsed in distilled water, air dried and a cover glass (POLAR) was mounted with DPX (BDH).

FPG STAINING: (Perry and Wolff, 1974 and Gota et al., 1974). The slides were stained for 15 minutes in a 50 μg/ml Bisbenzamide (Hoechst 33288- SIGMA) in phosphate buffer saline (PBS-GIBCO). At the end of this period the slides were rinsed briefly in distilled water and air dried. Three drops of PBS were added and the cover-glass was mounted. The sides of the slides were sealed with standard rubber solution (DUNLOP). The slides were then kept 10 cms. below a UV bulb (Phillips - 220 V; 100 W) for 45 minutes or in the sun light for
1 hour. They were then washed in distilled water and stained in 5% Giemsa solution in phosphate buffer (pH 6.8).

MICROSCOPY: The slides were scanned using a Leitz Orthoplan microscope. The metaphases were scanned using the 10X objective and chromosomes were analyzed using the 100X objective. For scoring the micronucleus, the slides were scanned using a 40X objective.

PHOTOGRAPHY: The metaphases were photographed using an automatic Leitz Orthomat camera with 'blue-green' filter. The film used was MA 8, 16 ASA (ORWO).

DEVELOPMENT: The film was developed using contrast developer (M & B) diluted 1:1 for 5 minutes at 20°C. The film was rinsed quickly in water and fixed with acid fixer (AMFIX) for 5 minutes and air dried.

PRINTING: The photographs were printed on single weight hard-paper (AGFA) using an enlarger (AGIL) and developed using the same developer and fixer.

3.4 Criteria for Scoring Chromosomal Aberration:
(Savage, 1975, ISCN, 1978 and IAEA, 1986)
Chromosomal aberrations are broadly classified into 3 main types:
(a) Chromatid Type: When one of the chromatid of a chromosome is involved in an aberration.

(b) Chromosome Type: When both the chromatid of a metaphase chromosome are involved in an aberration.

(c) Sub-Chromatid Type: When part of a chromatid (less than half the width of chromatid) is involved in an aberration (Kihlman and Natarajan, 1983). (Fig. 5).

CHROMOSOME-TYPE OF ABERRATIONS

Chromosome type of aberrations are further classified into various types:

Terminal and Interstitial Deletions (Breaks): For radiation exposure the terminal deletions are called acentric fragments if the fragment has paired chromatids. The interstitial deletions appear as two dots (minutes) and the bigger interstitial deletions in which there is a clear space in the centre of a ring are usually called acentric ring. Acentric fragments associated with interchanges between chromosomes are not classified as terminal deletions (IAEA, 1988). (Fig. 1, 2).
Asymmetrical interchanges: Chromosomes having 2 centromeres are called dicentric chromosomes. It is assumed that a dicentric chromosome at first in vivo metaphase will be accompanied by an acentric fragment. This acentric fragment is a result of dicentric formation, so it should not be scored separately. Tricentric chromosome is scored as two dicentric chromosomes and the metaphase having tricentric chromosome will usually have two accompanying acentric fragments. (Fig. 1, 6).

Symmetrical interchanges: If there is a break at two ends of a chromosome they might fuse to form a centric ring and an acentric fragment. This fragment should not be scored separately as acentric fragment. (Fig 2, 4).

Symmetrical Interchanges: It is usually difficult to observe these by conventional staining preparations, unless this interchanged pieces produce two chromosomes that are distinctly different from normal chromosomes. The analysis of this interchanges (reciprocal translocations) are time consuming and not recommended for radiation-induced aberrations.
Symmetrical Intrachanges: They are exchanges within the chromosomes. An intrachange within a chromosome involving the centromere is called paracentric inversion and an intra change not involving the centromere is called as a paracentric inversion.

**CHROMATID-TYPE OF ABERRATIONS**

Chromatid-type of aberrations are further classified as:

**Terminal and Interstitial Deletions (Breaks):** Terminal deletions are breaks at one end of a chromatid of a chromosome, there can be a displacement of the broken acentric single fragment or it can be lost. Interstitial deletion is a break in one of the chromatids anywhere except at the terminal end and the deleted acentric single fragment is usually deleted. There may be a gap between the centric part and the telomeric part of the chromatid and if this gap is bigger than the width of a chromatid, then it is called as a terminal deletion or a break. (Fig. 7, 8, 9a, 9b).

**Achromatic Lesions:** These are non-staining regions on a single chromatid of a chromosome. If this non-staining region is smaller than the width of a chromatid then it is called an achromatic lesion or a gap. Generally gaps
are recorded but not included in the total count of aberrations, since their significance and relationship to other 'true' aberration types are, at present not clear (IAEA, 1986). (Fig. 8, 9a, 9b).

**Iso-Chromatid Deletions (Breaks):** This is a break at the same loci on the sister chromatids. There are many types of iso-chromatid deletions eg: if the centromic part of the chromatids fuse, they are classified as proximal union, and if the broken ends or the telomeric ends of the sister chromatids are fused they are classified as distal sister union. In mammalian cells this sister union(s) are very rare, the usual once observed are of the non-proximal and distal type. While scoring radiation-induced chromosomal aberrations these iso-chromatid deletions are classified as terminal deletions (acentric fragments), since the spontaneous levels of these types of aberrations are very low, it is not unreasonable to classify this way (IAEA, 1986). (Fig. 9a, 9b).

**Chromatid Exchanges:** They are classified as inter arm inter change and asymmetrical chromatid exchanges. These exchanges are chromatid-type of dicentrics (IAEA, 1986). (Fig. 10).
**Symmetrical Interchanges:** In this type of interchanges the somatic pairing maintains an association between the chromosomes involved in the exchange.

**Triradials:** This is the interaction between a chromosome having an iso-chromatid deletion and a second chromosome having a chromatid deletion.

**Criteria for scoring SCEs:** Only metaphases having the complete set of chromosomes were chosen for scoring SCEs. Exchange of dark and light regions at the terminal end is taken as one exchange and one reciprocal exchange at the middle (interstitial) region of a chromosome is taken as two exchanges. Exchanges at the centromeric regions are not scored, as it is difficult to differentiate between a chromosome twist and an exchange in this region. For SCEs at least 50 metaphases were scored. (Fig. 3, 4).

**Criteria for Scoring Bi-nucleate micronucleus:** The cell should have two nuclei and a clearly defined cell boundary. Broken cells were not scored. The micronucleus should be stained darker than the nucleus and it should not be bigger than 1/5th the size of a nucleus. If two cells are fused intimately then they are not considered for scoring. At least 1000 cells were scored for each set of x-ray dose. (Fig. 11).
Analysis of Cell Cycle Kinetics: At least three EPG stained slides were scanned for each dose. Incomplete were not scored. 200 to 300 complete metaphases were scored for each dosage. The metaphases at different cell cycles were classified as follows:

(i) If both the chromatids of a metaphase chromosome are stained equally and all the chromosomes of a particular metaphase spread were stained uniformly, then the metaphase was considered as belonging to the First Cycle (M_1). (Fig. 1, 2).

(ii) If one of the chromatids of a metaphase chromosome is stained darkly and the other sister chromatid is lightly stained (harlequin effect) and all the chromosomes of that metaphase spread shows the same effect than the metaphase belongs to second cell cycle (M_2). (Fig. 3, 4).

(iii) If 3/4th of a chromosome is darkly or lightly stained and a few chromosomes of that particular metaphase spread also shows a 'harlequin' effect, then the metaphase belongs to Third or subsequent cell cycle (M_3+). It is not possible to distinguish between third and subsequent cell cycles (Scott et. al., 1977). (Fig. 6).
1 Metaphase showing dicentrics (---),acentric fragments (←) and interstitial deletion (←).
Fig. 3 Second Cycle metaphase showing 26 SCEs. Interstitial exchange (↔).

ring.
Fig. 5  Metaphase showing endoreduplication with sub-chromatid exchanges (←→).

CHROMOSOMAL ABERRATIONS IN THIRD CYCLE METAPHASES

Fig. 6  Third-Cycle metaphase showing dis...
Fig. 7 Metaphase showing chromatid breaks (deletions).
Fig. 9a Metaphase showing extensive fragmentation with chromatid, iso-chromatid breaks (→) achromatic lesions.

Metaphase showing extreme fragmentation with chromatid, iso-chromatid breaks and achromatic lesions.
Fig. 10: Metaphase showing chromatid-exchange (Quadriradial).
For assessing the cell cycle kinetics the proportion of cells at I, II or III or subsequent cell cycles were scored for a particular dosage and the period of incubation (48 or 72 hour culture). 200 to 300 metaphases were scored per dosage.

Criteria for Scoring Mitotic Index: Three slides were scored and at least 10 fields were chosen for assessing mitotic index for each dosage. In each field the number of blast cells and mitoses were recorded. At least 1000 blast cells were scored and the mitotic index was calculated as follows.

\[
\text{Mitotic Index} = \frac{\text{metaphases}}{\text{number of blast cells}} \times 100
\]

3.7 STATISTICAL ANALYSIS: The published results show evidence that the expected yield of aberrations (Y) is related to dose (D) by the equation:

\[
Y = A + \alpha D + \beta D^2
\]

The estimates of alpha and beta coefficients were determined by the formula as follows:

let: \[X_1 = D\] \[n = \text{sample size}\]
\[X_2 = D^2\] \[Y_1 = Y\]
\[X_3 = D^3\] \[Y_2 = YD\]
\[X_4 = D^4\] \[Y_3 = YD^2\]
The estimates are:

\[ \beta = \frac{(X_2y_2 - X_1y_3)(X_1^2 - nX_2) - (X_1y_1 - ny_2)(X_2^2 - X_1X_3)}{(X_1X_3 - X_1X_4)(X_1^2 - nX_2) - (X_1X_2 - nX_3)(X_2 - X_1X_3)} \]

\[ \alpha = \frac{(X_1y_1 - ny_2) - \beta(X_1X_2 - nX_3)}{X_1^2 - nX_2} \]

\[ A = \frac{y_1 - \beta X_2 - \alpha X_1}{n} \]

For X-rays the dicentrics yield follows Poisson distribution. The technique recommended for determining best-fit coefficients is that of maximum likelihood (Papworth, 1975). This can be done by maximizing the likelihood of the observation assuming Poisson distribution by the method of iteratively reweighted least squares. For over dispersed non-Poisson distributions, the weights must take into account the overdistribution. If data shows a trend of \( \sigma^2/y \) with dose, then this trend should be used. Otherwise, Poisson weights are divided by an average value of \( \sigma^2/y \).

\[ \sigma^2/y \] is calculated as follows:

mean \( y = \frac{\text{No. of aberrations in cells}}{\text{No. of cells}} \)

\[ = \frac{\sum_{i=1}^{N} (x_i - y)^2}{N} \]
\[ N \text{ = Total No. of cells} \]

\[ X_i \text{ is observed aberrations for 1 cell} \]

The standard errors of the coefficients and the fitted values are based on Poisson distribution or overdispersed distribution. If there is a lack of fit, then the standard error were increased by chi-square values divided by the degree of freedom raised to the power of \( 1/2 \) i.e. \((X^2/DF)^{1/2}\).

For comparing the mean of controls and diseases Student's 't' test was applied. Correlation coefficient between the age and the yield of aberrations for a given dosage has been computed and the regression equation has been obtained.

Analysis of variance 'F' test was used to compare the difference in the yield of aberrations for a given dosage among the diseases.