Materials and methods
Materials and Methods

The couples with history / complaints of early pregnancy loss of 20 weeks of gestation of age group between 20-40 yrs, who had been referred from the Out patient wing of Department of Obstetrics & Gynecology of JIPMER Hospital including nearby peripheral hospitals formed the material for the current study. The cases of early pregnancy loss included the cases with history of typical early pregnancy loss and cases of repeated pregnancy loss with more than two spontaneous first trimester abortions. Initially each couple was screened for chromosomal aberrations and subsequently for the involvement of damaged DNA through Comet Assay analysis and for Micronucleus score. A total of seventy two couples (one hundred and forty two individuals) were screened.

In addition, a total number of eighty age matched normal healthy couples (one hundred & sixty individuals) without any history of abortions/miscarriages during their life time were taken as controls for the above mentioned study. Informed consents were received from all the participants including the cases. All couples (both case & controls) were interviewed for information like reproductive history, past illness - surgical/medical interventions, metabolic disorders like diabetics, personal habits like consumption of alcohol, smoking of tobacco/cigars, exposure to chemicals, other genotoxic agents, occupational hazards if any were collected and entered in a data sheet - Proforma (Annexure). The control couples were also equally investigated for exposure to above said risk factors and the data was recorded.

The study was presented to Institute Research Council of JIPMER and also to Human Ethics Committee of JIPMER for approval and implementation. The guidelines framed and constituted were followed and the study was carried and completed from Dept. of Anatomy, JIPMER, Pondicherry.
Selection criteria for cases
Inclusion criteria
- Couples with two or more consecutive pregnancy losses due to unknown causes.
- Couples with history of still birth, malformed child, or a family history of the same.

Exclusion criteria
- Couples with pregnancy losses due to various known causes.

All cases and controls were subjected for the following

I. Cytogenetic Analysis by :-
   - Conventional lymphocyte cell culture
   - Karyotyping – Automation – using Software

II. Comet Assay –
   - for detection of DNA damage
     by Single Cell Gel Electrophoresis –SCGE
     Alkaline Comet Assay
   - Measurement of Comets using Software – Comet score

III. Micronucleus Score
   - for attributing chromosomal damage.
   - Subjecting the lymphocyte cell culture to Cytochalasine B
     Cytokinesis Blocked Micronucleus Assay –CBMN Assay
     Detection of binucleated cells – score
     Detection of Micronucleus in binucleated cells
     Detection of Nucleoplasmic bridge in binucleated cells
     Detection of Nuclear Buds in binucleated cells

<table>
<thead>
<tr>
<th>Nuclear Bud</th>
<th>indicative of Gene amplifications &amp; mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoplasmic bridge</td>
<td>indicative of dicentric chromosome</td>
</tr>
<tr>
<td>Micronucleus</td>
<td>indicative of Chromosomal or chromatid loss, acentric fragments of chromosome etc.</td>
</tr>
</tbody>
</table>
I. CYTOGENETIC STUDY & ANALYSIS

A. Setting of lymphocyte cell culture
   i. Preparation of culture media
   ii. Preparation of Serum for culture media
   iii. Setting up of Culture

B. Harvesting of chromosome

C. Preparation of slides and staining

D. Microscopy

E. Application of Automated Karyotyping software

A. Setting of lymphocyte cell culture

   i. Preparation of culture media

   1 packet RPMI 1640 (10.3 gms) (Sigma Aldrich) was dissolved in 95 ml autoclaved deionised water and made up to 100ml. This was further divided into 10 ml aliquots.

   To prepare 100 ml culture media, 10 ml stock solution was taken and mixed with 85 ml autoclaved deionised water and 5 ml of Na₂HCO₃ (1 ml / 100 ml (7.5 gms Na₂HCO₃ in 100 ml) and the pH was adjusted to 7.0 by adding dilute 1N HCl (10 ml diluted in 90 ml of autoclaved distilled water) in drops for reaching the desired pH. After preparing the working solution, the medium was filtered through the membrane filter – Millipore filter of 0.22 μm or 0.45μm millipore filters.

   Tissue culture grade of antibiotics – Penicillin-Streptomycin (0.75 gms dissolved in 2.5 ml of autoclaved water) and 0.25 μl of each is added to 100 ml media.
Recommended complete Medium for culture of Lymphocytes – RPMI 1640
(Rooney & Czepulkowski, 1986)

<table>
<thead>
<tr>
<th>Component</th>
<th>Strength</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640</td>
<td>1X</td>
<td>100.0</td>
</tr>
<tr>
<td>Fetal Calf Serum</td>
<td></td>
<td>20.0</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>200 mM (100X)</td>
<td>1.0</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>10,000 IU/ml +</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>10,000 μg/ml</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>7.5%</td>
<td>1.0</td>
</tr>
</tbody>
</table>

ii. Serum for culture media

Serum was added as a supplement to the culture media for achieving optimum cell growth.
The types of sera which are commonly used are:–

a. FCS- Fetal calf serum,
b. FBS- Fetal Bovine serum
c. NCS- New born calf serum (taken from animals 10 days old or less)
d. CS – Calf serum (taken from animals 10 weeks old or less).
e. Cord blood serum
f. AB +ve serum

20ml of FCS was added to 80ml of above said filtered RPMI 1640 culture media and made up to 100ml. This was further divided in aliquots of 5ml in 20 nos of flat bottomed screw capped borosil make glass culture vials.
iii. Setting up of Cultures

Lymphocyte cultures were set up by the modified method of Arakaki and Sparkes (1963) using PHA-M (Sigma Aldrich) as mitogen.
150 µl of PHA-M (Sigma Aldrich) was added to each culture vial of 5ml media and 0.5ml of whole blood from the cases was added to the above and the vials were then incubated for 69 hrs at 37°C.

B. Harvesting of chromosome

- After 69 hrs, 0.50µl of 3 mg % Colchicine was added.
- It was mixed gently and returned to the incubator for 30 minutes.
- Culture vials were removed from the incubator and the contents were transferred into centrifuge tubes.
- The tubes were centrifuged at 130 g (900 rpm) for 10 minutes.
- The supernatant was discarded.
- 10 ml of hypotonic (0.075M KCl) solution pre warmed to 37°C was added to the pellet while agitating the tube with whirl mixer.
- The tubes were incubated at room temperature or inside the incubator for 20 minutes.
- The tubes were centrifuged for 10min at 900rpm.
- The supernatant was discarded and 10ml of cold, freshly prepared fixative (3 parts methanol : 1 part glacial acetic acid) was added by drops with constant mixing.
- The tubes were centrifuged after half an hour at 900 rpm for 10 minutes.
- Two more washings of the cell pellet with fixative was carried out, until a white cell pellet was obtained with clear fixative is seen.
- The tubes were centrifuged and the supernatant discarded leaving approximately 0.5 ml above the cell pellet. The cells were mixed well.
C. Preparation of slides and staining

For making of slides the method of Moorhead et al. (1960) with a slight modification as per Spurbeck et al. (1996) was followed. The fixed cell pellet thus obtained were loaded into a Pasteur pipette. Two or three drops of the cell suspension was dropped from a height of 1 to 2 feet onto the wet & chilled slide at 20-30° angle. Immediately the slides were exposed over a hot water bath for 5-6 seconds to achieve exposure of 45% humidity condition on surface of slide (Lundsteen and Lind; 1985). This helps to prevent overlapping of chromosomes and in spreading of chromosomes effectively. Three to four slides are normally made from each sample. The slides were left to dry on the hot plate at 43°C.

Giemsa banding (GTG banding)
The analysis was performed on G-banded chromosome preparations.

Principle: The slides are dipped in protein digesting enzyme (trypsin) for appropriate time followed by staining with Giemsa stain. On microscopic observation the bands are revealed as alternate dark and light bands which are characteristic for each chromosome. A resolution of 400-550 bands per haploid karyotype was achieved as a minimum.

Procedure:
To obtain various dark and light bands of GTG banding in chromosome, method of Seabright M. (1971) was followed which is the most accepted and commonly followed method for staining/bANDING mammalian chromosomes. The slides were left for 2-3 days at room temperature prior to trypsinisation. Trypsin (1:250; GIBCO BRL) working solution was prepared in PBS was used. The slides were
first dipped in trypsin for 20-30 seconds followed by rinsing with deionised water and finally placed in Giemsa stain solution for 3 – 4 minutes. After staining, the excess stain on the slide was washed with tap water and left for air drying.

D. Microscopy

Slides were observed with bright field microscope under 20X initially followed by 100X magnification later. After the initial analysis, a well banded spread was grabbed into the computer using a CCD camera with the help of Karyotyping software – Ikaros from Metasystem, Germany for identification of each chromosome and for placing them at appropriate place for final format of Karyotyping.

E. Application of Automated Karyotyping software “Ikaros”

The Ikaros karyotyping work station consist of the following

I. Hardware

II. Software

I. Hardware

The components of the hardware are mainly a high end Desktop system – central unit and an image acquisition unit.

Central unit: The central unit of automated Karyotyping system is a computer (IBM, Germany), typically equipped with an Intel® Pentium® 4 Processor (1.2 GHz), 512 MB RAM memory, and an 80-GB hard drive and the operating system Microsoft® Windows® 2000. For data archiving purposes the Ikaros system is equipped with a magneto-optical disk drive (Olympus 2.3 GB capacity). Ikaros software requires a three-Button-Mouse to perform all important tasks comfortably.
Image acquisition hardware: For image acquisition a high-resolution monochrome megapixel charge coupled device (CCD) camera (JAI CV-M300; JAI AS, Glostrup/Copenhagen, Denmark) with a resolution of 768 x 576 pixels (2/3) CCD; Pixel size 6.7 μm x 6.7 μm; signal-to-noise ratio 56 dB) is used. It is connected to a gray level digitizer board (yncrol digitizer) installed in the central unit, which provides real-time digitization of video signals at a resolution of 1280 x 1024 pixels with 256 gray levels. The camera is connected to the microscope via a standard 1.0× TV adapter (C-mount 60 C, 2/3); Carl Zeiss, Göttingen, Germany).

Microscope: Ikaros autokaryotyping system is connected to the Trinocular Research microscope BX51 (Olympus, Japan) which is a high end microscope in this category, takes full advantage of the microscope components for light source adjustment for bright-field imaging and Photomicrography.

II. Software

MetaSystems' automated Karyotyping system includes the software modules both Ikaros & Isis for on-screen karyotyping, analysis and reporting of Chromosomal abnormalities as described below.
Figure:1. Description of *Ikaros* – Indigenous Karyotyping software from *Metasystem*, GMBH

*Ikaros* is an ingenious software package with an intuitive graphic user interface with a variety of powerful tools to provide the flexibility needed for the analysis of even the most complex karyotypes. *Ikaros* handles G-, R-, C- and Q-banded chromosomes as well as polyploid cells in brightfield, phase contrast, and epifluorescence. Ikaros complies with GLP standards allowing any individual for operation to be reviewed and corrected at any time.
Image Acquisition and Metaphase processing

Equipped with a high resolution CCD camera, Ikaros acquires high quality images in seconds. The live image is displayed on screen during the capture, and computer-assisted microscope adjustment assures optimum image quality. The multiple fields capture tool facilitates the acquisition of large metaphase spreads, which may be combined to one image, or just left as they are. Several tools for automatic and interactive multiple level image enhancement, a masking function for fast cleanup of the image area.

Various options allow automatic enhancement of the images subsequently to the capturing procedure. Contrast can be adjusted by selecting the object threshold and there by to achieve excellent banding pattern in banded slides, for which the filter power is set in real time with mouse. Along with image enhancement procedure, the software automatically counts the chromosomes and displays the same. Automatic as well as semi-automatic and manual functions with auto zoom facilities of the software help the separation of chromosomes which are attached, overlapped or clustered. Thus it provides highest possible precision and efficiency in isolating the chromosomes of complicated spreads like spreads of prometaphase stage of chromosomes.

The original image and all processing steps are stored on disk, enabling unlimited verification and correction of any step. Any work with the image can be done either in the original metaphase, the preprocessed image or the processed (means: finally separated) metaphase. Metaphases can be displayed in three different stages of the image processing, the original, pre-processed and processed metaphase.

Flexible annotation functions in combination with customizable printing templates allow to create professional reports.
From interactively pre-classified metaphase *Ikaros* will automatically create Karyotype with a single mouse click by importing the chromosomes from each image field for karyotyping.

**Karyotyping**

In the karyotype view, automatic and interactive chromosome classification and Karyotype generation for G-, R-, Q-, C-, and DAPI-banded chromosomes are provided. During chromosome assignment or Karyotyping, the selected chromosome is simultaneously gets displayed in the metaphase context, supports multiple capture fields. Once assigned, the chromosomes may be straightened, shifted, rotated, resized and mirror image can be created and also automatically aligned at the centromeres and displayed side by side with ISCN banding ideograms. An appealing Karyotype can be generated by single or multiple chromosome contrast modifications. Ikaros is supported with Ideograms (diagrammatic representation of a Karyotype) which provides the details of band and sub-band numerical designation as of ISCN specifications/standard (G-,R-banding). It also offers to designate the areas interest with different shades of colors to identify the rearranged ideograms for the documentation of chromosomal aberrations.

The system supports various karyotype templates, for human metaphases and many other species. Composite karyotypes are automatically created for the direct comparison of homologous chromosomes from different patients.
Operational procedures of the Auto karyotyping software.

1. Capturing the image of a metaphase spread into the system.
   
   To achieve this, the shutter exclusively meant for the transfer of image from the microscope should be released. Thus the image falls over the electronic sensor of the camera which will be transferred to the system by the grabber card.

2. Freeze the image by right click, after adjusting the focus and intensity of light.

3. If the entire Metaphase spread is in a position to be captured, the above said step generally initiated.
   
   In some, if any of the chromosome/s is/are found beyond the limits of the given territory of the spread, such a chromosome/s which is/are to be included is done by clicking “Add capture”.

4. In the next step adjust the threshold of the captured image by “Object threshold” option. This will enable to increase/decrease the contrast of the chromosome bands.

5. If there is any foreign body or whole lymphocyte cell included in the vicinity of the captured field, the option of “Mask Meta” may be applied [click and draw a boundary around the spread] to specify the boundary of the Metaphase spread.

6. If the Metaphase spread is without any overlap of chromosomes the tool “check” is used to ascertain no overlapping. If it is a whole spread with individual chromosomes, make Karyotype by clicking the cursor/arrow over the karyo-form at the right upper corner of the Window.

7. If there is any overlap of chromosomes/two chromosomes are lying side by side and if the software has treated it as a single chromosome. Then the tool “Overlap” or “Separate” may be used for separating those chromosome. After that once again the number is checked and confirmed. If satisfactory, the command karyotype is used.
ANALYSIS AND INTERPRETATION

All spreads/karyotypes and chromosomal abnormalities were analysed and determined in accordance with the International System for Human Cytogenetics Nomenclature (Felix Mitelman; ISCN 1995).

Classification of chromosomes

The chromosomes as seen in a metaphase spread are tightly coiled packages of DNA and protein. They consist of two sister chromatids that would have separated at anaphase and ended up in separate daughter nuclei had they not been fixed. The primary constriction – centromere is the attachment site of spindle fibers. This divides the chromosomes into a short arm and a long arm. The free end of the arm is called the telomere. Based on the position of the centromere the chromosomes are classified as metacentric (centromere in the middle), telocentric (centromere right at the end), acrocentric (centromere very near to the end and with a very short arm., the short arms may be usually satellite) and submetacentric (the centromere is slightly away from the center resulting in a short p arm and a long q arm). The length of the human metaphase spreads is very much dependent upon the method of preparation, the average size of the longest chromosome is about 10 μm and the smallest is about 2 μm.

A conventional scheme for classifying the human chromosome was developed in the early 1960s which is summarised in the International System for Human Cytogenetic Nomenclature (1995). The unbanded preparations are used for preliminary analysis. Based on the size and position of the centromere the human chromosomes of an unbanded preparation can be classified as follows:-
Group A
(no. 1 – 3) Large metacentric (no. 1 & 3) or sub metacentric (no. 2) chromosomes readily distinguished from each other by size and centromere position;

Group B
(no. 4 – 5) Large sub metacentric chromosomes which are difficult to distinguish from each other;

Group C
(no. 6 – 12 – X) Medium sized submetacentric chromosomes. The X chromosomes resembles the longer chromosomes in this group. It is difficult to classify this group with out banding;

Group D
(no. 13 – 15) Medium sized acrocentric chromosomes with satellites.

Group E
(no. 16 - 18) Relatively short metacentric chromosomes (no. 16) or submetacentric chromosomes (nos. 17 & 18);

Group F
(no. 19 - 20) Short metacentric chromosomes;

Group G
(no. 21 – 22 - Y) Short acrocentric chromosomes with satellites. The Y chromosome is similar to these chromosomes but bears no satellites.

Banding is done on aged (2 - 3 days) slides and a band is defined as that part of a chromosome which is clearly distinguishable from its adjacent segments by appearing darker or lighter with one or more banding techniques. The chromosomes are visualized as consisting of a continuous series of light and dark bands, so that, by definition, there are no interbands. Original Banding pattern was described in the Paris Conference (1971) report and based on the pattern observed in different staining method, Q-, G- or R- banding technique. The best cell to analyse usually have between 400 and 600 bands per haploid karyotype [ISCN (1981)], depending on the indications of analysis. Bands serving as landmarks on each chromosome is described in Annexure (Table: CG:IX)
The location of a particular spread is made by recording the coordinate readings of both the vernier scales of the stage micrometer of the microscope and the readings are noted. In conditions/circumstances of a need for a recall of the same spread, the said reading would be of much help to get back to the field of interest.
Metaphase view of Ikaros karyotyping software showing captured metaphase spread – G band
Karyotype view of Ikaros karyotyping software showing a G band Karyotype
II. SINGLE CELL GEL ELECTROPHORESIS ASSAY - "COMET ASSAY"

The single-cell gel electrophoresis (comet) assay is a sensitive technique to detect damaged DNA at the individual cell level and specifically for DNA strand breaks. The principle of this assay is based upon the ability of denatured and cleaved DNA fragments to migrate out of the cell under the influence of an electric field. Undamaged DNA remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail which is produced from fragmented DNA shape and migration pattern allows for assessment of DNA damage. In this assay cells are immobilized in a bed of low melting point agarose. Following gentle cell lysis, samples are treated with alkali to unwind, denature the DNA, and hydrolyze sites of damage, and then subjected to electrophoresis. The cells are then stained with a fluorescent DNA intercalating dye. The sample is visualized under the microscope by epifluorescence. As an alternative, silver staining allows standard light microscopy to be used for data analysis. The comet assay has been used in various studies to investigate the effect of Reactive Oxygen Species on DNA, and the protective effects of certain dietary antioxidants. When peroxides are used the assay becomes an assessment of residual antioxidant activity which prevents peroxide attack on DNA. It is increasingly used in genotoxicity testing as well as in human biomonitoring studies.

Cases & Controls

The said couples and controls were assessed for DNA damage studies which were carried out in the Cytogenetic lab of department of Anatomy. The healthy normal controls were between 21 and 32 years of age.

For the purpose of Comet Assay 2-3ml Heparinized blood was collected from all the individuals within one – two weeks after the loss of pregnancy.
“COMET ASSAY” - Steps involved (Saran & Ahuja, 1999)

I. Separation of lymphocyte

II. Preparation of slides
   1) Preparation of Agarose
   2) Layering of gel - Agarose precoating
   3) Layering of Lymphocyte-Agarose gel mixture
   4) Layering of the third layer of gel

III. Lysis of lymphocyte (micro-gel-slide)

IV. Alkaline unwinding of DNA

V. Electrophoresis of slides

VI. Neutralisation

VII. Staining

   Fluorescent Staining

   Silver Staining Method
      i. Drying
      ii. Fixation
      iii. Washing & Drying
      iv. Silver Nitrate Staining
      v. Stopping of staining
      vi. Rinsing with water

VIII. Microscopy

IX. Evaluation of DNA damage
I. SEPARATION OF LYMPHOCYTE

2ml of peripheral venous blood was collected from both the cases and controls. Lymphocytes were separated (Boyum, 1974) from the blood using Histopaque® and separated lymphocytes are suspended in phosphate buffered saline.

II. PREPARATION OF SLIDES

Plain grease free, clean microscope slides are used for layering the gels.

Step I: Preparation of Agarose

0.5% low melting point agarose (LMPA) and 0.75% normal melting point agarose (NMPA) are prepared in PBS.

Step 2: Layering of gel - Agarose precoating

For making the first layer of gel, onto a clean, dry, plain glass micro-slide about 150µl of hot, 1% NMPA is dropped and smeared in one direction with the help of another plain slide inclined at about 45°C. These slides are allowed to dry at 37°C overnight.

Step 3: Layering of Lymphocyte-Agarose gel mixture

Once NMPA is set, 75µl LMPA (37°C) mixed with 20µl of whole blood/10 µl of lymphocyte cell suspension, is added onto the NMPA layer. Put a cover slip without making air bubbles and the gel is allowed to set at 4°C for 5-10min.

Step 4: Layering of the third layer of gel

For the third agarose layer, the cover slip is gently removed and 75µl of LMPA is added onto the second agarose layer. The cover slip is replaced and agarose is allowed to set again at 4°C for 5-10min.
III. Lysis of Lymphocyte (micro-gel-slide)
The cover slip is removed again and the slides are gently lowered into cold lysis solution and refrigerated for minimum 1h. The lysis treatment may extend up to a maximum of 24h.

All the above steps were performed under dimmed yellow light to prevent additional DNA damage.

IV. Alkaline unwinding and Electrophoresis of slides:
Step1: After lysis at 4°C, the slides are gently removed from the lysing solution and placed side by side in horizontal electrophoretic box. The slides are placed as close together as possible, with their gels touching each other.

Step2: The electrophoretic reservoir is filled with fresh electrophoretic buffer until the buffer level completely covers the slides with out the formation of air bubbles over the agarose gel.

Step3: Alkaline unwinding
The slides are allowed to stay in the alkaline buffer for 30min to allow unwinding of DNA and the expression of alkali labile damage.

V. Electrophoresis
Step4: Power supply is turned on to 20V (PLATE:3) and the current is adjusted to 300m Amp by raising or lowering the buffer level. Electrophoresis is carried out for 30min.

Step5: The power supply is turned off.

Steps 1-4 must be performed under dimmed yellow light.
PLATE: 3
Electrophoresis of Microgel slides

Photograph showing Electrophoresis of Comet slides – Microgel slides, using submarine tank and Power pack (BIORAD®) at 20V
VI. Neutralisation
The slides are gently lifted from the buffer and placed on a staining tray. The slides are carefully flooded with neutralizing buffer (pH7.4) for 10 minutes and the buffer is drained; the process is repeated two more times.

VII. STAINING
VIII-A: Fluorescent staining Method
To each slide, 50μL of 1X Ethidium Bromide stain is pored and the slide is covered with a clean cover slip. Before viewing the slides, excess stain from the back and edges of the slides may be blotted away.

Microscopy
For visualization of ethidium bromide stained slides, a fluorescent microscope equipped with an excitation filter of 515-560nm and a barrier filter of 590nm, a magnification of 250x is used.

VIII-B: Silver Staining Method

I. Solutions required
   (A) Fixing solution
   (B) Staining solution – I
   (C) Staining solution – II
   (D) Working staining solution {I+II}
   (E) Stopping solution

II. Procedure

Step 1: After electrophoresis and neutralization the gel slides are dried for about an hour at room temperature.
Step 2: These slides are placed in fixing solution (A) for 10min and after that washed several times with H2O.

Step 3: The slides are then allowed to dry at 37°C for at least 1h to a maximum of overnight.

Step 4: Staining of slides
For staining the slides, fresh staining solution (D) is poured gently over the slides and cover/flooded all slides uniformly. The tray containing the slides was shaken very gently throughout the staining procedure of 10-20min to ensure uniform staining. This step was repeated a couple of times with fresh staining solution(D) until a grayish colour develops on the slides.

Step 5: the slides were then transferred to a tray containing the stopping solution(E) for about 5min or until a yellowish brown colour develops. The slides were then washed again with H2O and allowed to dry in inclined position at room temperature.

Microscopy
The slides stained with Silver Nitrate may be observed under a transmission/bright field microscope.

IX. EVALUATION OF DNA DAMAGE

DNA damage may be estimated as length of the comet tail which is measured using an ocular fitted in the eyepiece of the microscope. Alternatively image analysis software has been used to quantitative additional DNA damage parameters such as tail area and tail moment ((length density). In total, 40-50 randomly selected cells are analyzed per sample.
Comet Analysis by Image Analysis Software - CometScore™

For the analysis of the comets the slides were scanned and 50 good comets were randomly selected from each slide and they were photographed and the resultant picture was processed with a software called CometScore™ (Version 1.5 from TriTek Corp.). This comet scoring software works from stored bitmap images of comet fields. The metrics which the software generate during the analysis were stored in a tab-delimited text file compatible with most third-party spreadsheet packages.

Calibrating the Software

The first step in Measuring Comets with Comet Score™ is Calibration of the software. For calibrating the software Capture an image of a ruler (Stage Micrometer) with the microscope camera at the same magnification that comets were captured and saved the image in the hard disk as bitmap (.bmp) file. This ruler image file later opened in the Comet Score™ software and set the Calibration by adjusting the size (length) of the calibration reticule. Later the value of the scale in the image entered in the reticule. The value of the scale should be entered in millimeters (10μm i.e. 0.1mm in the present study :- PLATE: 4 & 5 ).

After calibration the software measure comets in realworld units allowing data to be compared across multiple platforms, i.e., microscopes of different configurations.
Photograph of Ocular and stage micrometers used for the measurements of Comets and Calibration of the software
Photograph showing Calibration of the CometScore® software
Scoring Data

1. Open to load the comet image into the CometScore™.

2. Left-click near a comet and drag a rectangle around it. Release the button.

3. Position the cursor inside the rectangle at the center of the head and click the left mouse button.

4. Enter a filename to save the data for this comet (and all subsequent measurements until a new file is specified).

5. Left-click and drag a rectangle around another comet and indicate the center of the head with another left-click. This may be repeated as many times as needed to score as many comets in the field as desired.

6. If another image from the same case to be scored open the comet image into CometScore™ and begin scoring comets in this field to append the data to the previously specified data file.

7. To score the data from another case, open to load comet image from that case into CometScore™ and begin scoring comets and “Save As” to specify a new data output file before scoring any comets.
Comet Metrics

The following diagrams show some of the measurements that are applied to each comet.

Figure: 2. Illustration of Comet metrics can be measured with CometScore® software from AutoComet.com
Comet Metrics from Comet Score

1. Comet - any adjoining location in designated blob containing DNA — (conjoining pixels after segmentation)
2. Comet Length number of pixels in horizontal direction in Comet
3. Comet Height number of pixels in vertical direction in Comet
4. Comet Area number of pixels in Comet
5. Total Comet Intensity sum of pixel intensity values in Comet
6. Mean Comet Intensity mean intensity of pixels in Comet
7. Comet Profile vertical summation of pixel intensities in Comet
8. Head resulting profile from mirroring the comet profile about a designated center point
9. Head Diameter number of pixels in horizontal direction in Head
10. Head Area number of pixels in Head
11. Total Head Intensity sum of pixel intensity values in Head
12. Mean Head Intensity mean intensity of pixels in Head
13. Head Profile vertical summation of pixel intensities in Head
14. %DNA in Head Total Head Intensity divided by Total Comet Intensity (multiplied by 100)
15. Tail resulting profile from subtracting the Head Profile from the Comet Profile
16. Tail Length Head Diameter subtracted from Comet Length
17. Tail Area number of pixels in Tail
18. Total Tail Intensity sum of pixel intensity values in Tail
19. Mean Tail Intensity mean intensity of pixels in Tail
20. Tail Profile vertical summation of pixel intensities in Tail
21. %DNA in Tail Total Tail Intensity divided by Total Comet Intensity (multiplied by 100)
22. Tail Moment %DNA in Tail multiplied by Tail Length
23. Olive Moment summation of Tail Intensity profile values multiplied by their relative distances to the Head Center, divided by Total Comet Intensity
III. Cytokinesis Blocked Micro-Nucleus Assay

Micronuclei (MN) result from small chromosome fragments which are not incorporated into the main nuclei during cell division. They are enveloped by the nuclear membrane and appear as small nuclei outside the main cellular core. MN are mainly induced by aberrant chromosome fragments, which typically arise during exposure to various DNA damaging agents. Therefore they are widely accepted as an endpoint for fast DNA damage estimation (Fenech and Morley, 1985). Currently, the in vitro MN assay is widely used for the screening of various cell and DNA damages (Fenech et al., 1999) and for the estimation of genetic predisposition in cancer patients (Rothfuss et al., 2000).

Procedure

Culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum 2mM L-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin and 1.5% Phytohemagglutinin was chosen for conventional lymphocyte cell culture using peripheral venous blood. The lymphocytes were stimulated with PHA and after 44 hrs Cytochalasin-B (4.5ug/ml) was added and incubated for another 28 hrs (Fenech and Morley;1985, Sian Ellard and E M.Parry 1993) . Cytochalasin B (C_{29}H_{37}NO_{5}) M.W. 479.6. soluble in Ethanol and DMSO is a fungal metabolite, which acts as a microtubule inhibitor. It also induces cellular DNA fragmentation.

Cytochalasin B prevents the cells from completing the stage of cytokinesis and thus results in the formation of binucleated cells. The cells were harvested after 28 hrs. Three slides were prepared from each sample of cell pellet (Tung-Kwang
Lee;1994). The slides were air-dried and stained with plain Giemsa stain and examined at 20X and later at 100X magnification

**Brief procedure:**

1. About 5 ml of peripheral venous blood was collected in a heparinised vacutainer.

2. To 4.5 ml of RPMI 1640 supplemented culture media vial, 0.5 ml of whole blood was added.

3. Mixed and kept in an incubator at 37°C for 44 hrs.

4. About 3μg/ml of Cytochalasin B was added to each sample of 5 ml media and mixed well.

5. Further maintained at 37°C till 72 hours (from the time of setting up the culture).

6. The samples were centrifuged for 10 minutes at 800 rpm (250 x g).

7. For each cell pellet, about 5 ml of 0.6% KCl was added and centrifuged for 10 min at 800 rpm(250 x g).

8. Supernatant was discarded and the cell pellet was fixed with 10 ml of freshly made chilled fixative (Methanol: Acetic acid- 3:1) and kept in a refrigerator for 30 min.

9. The samples were again centrifuged at 250 x g and fixed twice with the fixative.

10. After discarding the supernatant the cell suspension was made 0.5 ml to 1.0 ml with fresh fixative.

11. The cell suspension was aspirated in to a pasteur pipette and dropped over a clean, chilled & wet slides.

12. The slides were air dried and stained with 2% Giemsa stain and observed under bright field microscope at 20X and100X magnification.
I. Criteria for scoring micronuclei (MNI)

a) Features of Binucleated cells

The criteria followed for cells that were subjected with cytokinesis-blocked agents for purpose of calculation of MN frequency were.

- The cells should be of binucleated form;
- The above stated nuclei should have an intact nuclear membrane
- The two nuclei of a binucleated cell should be of in equal size approximately, (including staining pattern and intensity);
- The two nuclei within a BN cell if attached by a fine nucleoplasmic bridge, the width should not be more than 1/4\textsuperscript{th} of the diameter of the nucleus.
- A cell with two overlapping nuclei might give a false positive sign of being binucleated. Hence the two distinct nuclei in the same plane either connected or not connected (with NPB / without NPB) is of utmost importance.
- The cell membrane of a binucleated cell should be intact and clearly distinguishable from the adjoining healthy cells.

The types of binucleated cells with various forms mentioned in the text are illustrated in the Figure no: 3.

Binucleated cells which can be scored for MNI

![Binucleated cells](image)

Figure:3. Criteria for choosing binucleate cells in the cytokinesis-block micronucleus assay. A. ideal binucleate cell; B. Binucleate cell with touching nuclei; C. Binucleate cell with narrow nucleoplasmic bridge between nuclei; D. Binucleate cell with relatively wide nucleoplasmic bridge. Cells with two overlapping nuclei may be considered suitable to score as binucleated cells if the nuclear boundaries are distinguishable. Occasionally binucleated cells with more than one nucleoplasmic bridge are observed.
b) *Features of nucleoplasmic bridges in binucleated cells*

Nucleoplasmic bridges are sometimes observed in binucleated cells following exposure to clastogens. These bridges are a continuous link between the nuclei in a binucleated cell and are thought to be due to dicentric chromosomes in which the centromeres were pulled to opposite poles during anaphase. The width of a nucleoplasmic bridge may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell. The nucleoplasmic bridge should have the same staining characteristics of the main nuclei. On very rare occasions more than one nucleoplasmic bridge may be observed within one binucleated cell. A binucleated cell with a nucleoplasmic bridge often contains one or more micronuclei. Examples of binucleated cells with nucleoplasmic bridges are illustrated in figure 1, 4 and 7.

c) *Features of Micronuclei in binucleated cells*

Micro nuclei (MNi) are morphologically identical to that of main nuclei but smaller in size; the features of which are:

- The diameter of MNi in human lymphocytes usually varies from 1/16th to 1/3rd of the mean diameter of the main nuclei.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.
- MNi are non-refractile and they can therefore be readily distinguishable from artifacts.
- MNi are not linked or connected to the main nuclei;
- MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the boundary of the main nuclei.

The various forms of MNi that meet the criteria set above are shown in Figure: 4.
Micronucleus that meet the scoring criteria

Figure 4. Typical appearance and relative size of micronuclei in binucleated cells. [A] Cell with two micronuclei one with 1/3rd and the other 1/9th the diameter of one of the main nuclei within the cell. [B] Micronuclei touching but not overlapping the main nuclei. [C] A binucleated cell with nucleoplasmic bridge between main nuclei and two micronuclei. [D] A binucleated cell with six micronuclei of various sizes; this type of cell is rarely seen.

Cellular structures that resemble MNi but should not be scored as MNi

Figure 5. Occasionally binucleated cells (or cells that resemble binucleated cells) may contain structures that resemble micronuclei but should not be scored as micronuclei originating from chromosome loss or chromosome breakage. These situations include [A] A trinucleated cell in which one of the nuclei is relatively small but has a diameter greater than 1/3 the diameter of the other nuclei; [B] dense stippling in a specific region of the cytoplasm; [C] extruded nuclear material that appears like a micronucleus with a narrow nucleoplasmic connection to the main nucleus and [D] nuclear blebs that have an obvious nucleoplasmic connection with the main nucleus.
d) **Conditions which give false positive signals for micronuclei**

*E.g. Multinucleated, Apoptotic, and Necrotic cells (Figure 6)*

**Apoptotic cells:**

- "Early" apoptotic cells - cells with chromatin condensation and intact cytoplasmic and nuclear boundaries.
- "Late" apoptotic cells - cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane often manifest as being of micronuclei.

**Necrotic cells:**

- "Early" necrotic cells - cells exhibiting a pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and damaged cytoplasmic membrane with a fairly intact nucleus.
- "Late" necrotic cells - cells exhibiting loss of cytoplasm and damaged/irregular nuclear membrane with scanty nuclear material also appear to be of micronuclear forms. Hence regarded as of false positive picture of a micronuclei.
Figure 6. The various types of cells that may be observed in the in vitro cytokinesis-block micronucleus assay excluding binucleated cells. These cell types shown should not be scored for MN frequency: [A] viable mono-, tri- and quadrinuclear cells; [B] mono- and binucleated cells at early stage of apoptosis when chromatin condensation has occurred but nuclear membrane has not disintegrated and late stage apoptotic cells with intact cytoplasm, no nucleus and apoptotic chromatin bodies within the cytoplasm; [C] cells at the various stages of necrosis including early stages showing vacuolisation, disintegration of cytoplasmic membrane and loss of cytoplasm with an intact nucleus and late stages in which cytoplasm is partially or completely lost and nuclear membrane is visibly damaged and nuclear material is commencing to leak from the remnant nucleus.

**Scoring Procedure**

The slides thus prepared were finally screened as follows for score purpose and also to interpret the role played by MNi leading to genomic instability.

- number of mononucleated (MONO) cells in 500 viable cells
- number of binucleated (BN) cells in 500 viable cells
- number of multinucleated (MULT) cells in 500 viable cells

Necrotic or apoptotic cells were not scored when determining the proportion of MONO, BN and MULT cells.
To determine the occurrence genomic instability in cells, the following were considered:

- the number of BN cells containing one or more micronuclei in 500 BN cells
- the total number of micronuclei in 500 BN cells
- the number of BN cells containing one or more Nucleoplasmic Bridges (NPBs) in 500 BN cells

In the case of a BN cell that has both a micronucleus and an NPB, the recordings were done independently.

Statistical methods

"One-way Analysis of Variance (ANOVA) with Dunnett's post test was performed using GraphPad InStat version 3.06 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com" The results were expressed as means ±SD. Linear Correlation(Pearson) were performed to establish the relations between the data obtained from each method of study.