

A RELIABLE BIOMARKER TO QUANTIFY GENOMIC INSTABILITY IN LYMPHOCYTESA. Manoj¹, B. Vishnu Bhat², C. Venkatesh² and Z. Bobby³

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ABSTRACT

Cytokinesis-Block Micronucleus (CBMN) Assay is a promising method to detect Double strand breaks of DNA and Chromosome Instability in somatic cells. This assay has been extensively used to evaluate the presence and the extent of DNA and chromosome damage in human populations exposed to Free radicals scavengers and genotoxic agents in various occupational settings, in the environment, or as a consequence of lifestyles. The technique has been done in dividing cell in invitro culture by blocking karyokinesis and cytokinesis with Cytochalasin-B in order to check whether the double strand break of DNA in the form chromatid breaks or mal-segregation of chromosome. Mutations of chromosome includes lagging of whole chromosomes, acentric chromosome fragments which is attributed by mutations of kinetochore proteins, centromeres and spindle apparatus leads to Micronucleus formation. Micronuclei are morphologically identical to nucleus but smaller with similar staining intensity as nucleus within an intact cytoplasm. The micronucleus index (MNI) for each sample was analysed by counting 1000 binucleated cells by manual method based on the scoring criteria framed by International collaborative project on micronucleus frequency HUMN project.

KEYWORDS: Cytokinesis-Block Micronucleus (CBMN) Assay, Cytochalasin-B, Micronuclei index(MNI).**INTRODUCTION**

The DNA is the blue print of our life which provides information for building all the proteins within every living things on the Universe. Its integrity is under constant attack by endogenous sources such as free radicals generated during oxygen deprivation and exogenous agents including chemicals and radiation. The accumulation of DNA damage can induce alteration in protein synthesis leads to Single strand adducts which is corrected by DNA repair pathway which restores the integrity of the DNA during cell cycle check points and the fidelity of repair is of great importance to the fate of cells. However in some cells SSB could not be repaired by intrinsic mechanism leads to double strand break with extensive DNA damage result chromosomal instability which cannot be counteracted by repair mechanism leads to irreversible growth of cell and it triggers apoptosis. Micronucleus has been originating due to chromosomal loss or mal-segregation of whole chromosome, acentric fragments (AF) due to double strand breaks during anaphase of cell division leads to chromosomal instability and these displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane and, except for their smaller size, are morphologically similar to nuclei which can be detected by Cytokinesis block Micronucleus (CBMN) Assay. The other anomalies such as nucleoplasmic bridges (NPB) and nuclear buds (NUB) due to dicentric chromosomes (DC) which may occur due to mis-repair of chromosome of DNA breaks at

telomeric ends producing fusion of two chromosome with two centromere which has also been detected in CBMN assay. Therefore CBMN assay is a best tool to evaluate DSB and chromosomal instability by adding cytokinesis inhibiting Cytochalasin-B at anaphase stage on 44th hour after incubation of cells in culture media in which any severe deformity of the DNA will appear as Micronuclei in the binucleated cell (BNC) during scanning.

Micronuclei assay was first proposed by Countryman and Heddle in 1976 in which they states that the characteristics micronuclei produced in cultured human lymphocytes arise from chromosomal fragments that are not incorporated into daughter nuclei at mitosis.^[1] In 1985 Fenech and Moorley improved the assay by evolving the techniques of Cytokinesis block micronucleus (CBMN) method in order to score MNI specifically in cells that had completed cell division which are recognised by their appearance as binucleated cells.^[2] Multiple molecular mechanisms that are thought to cause the formation of MN and other nuclear anomalies such as nucleoplasmic bridge (NPB) and nuclear bud (NBUD) due to mis-repair of DNA breaks.^[3] In lymphocytes, MN increase with age and are generally higher in females relative to males.^[4] Sex chromosomes contribute the majority of chromosome loss events with increasing age.^[5] In females, the X chromosome can account for up to 72% of the observed MN of which 37% appear to be lacking a functional kinetochore suggesting

that defects may be present in kinetochore assembly possibly due to X chromosome inactivation.^[6] The propensity for mis-repair of DNA breaks is enhanced if the error-free homologous recombinational DNA repair pathway is dysfunctional due to defects in relevant genes such as BRCA1 and BRCA2 furthermore, DNA breaks, which lead to MN formation, may be left unrepaired if repair enzymes in the non-homologous end joining pathway are defective.^[7] MN formation from acentric fragments include simultaneous excision repair of damaged or inappropriate bases such as uracil which is incorporated in DNA that are in proximity and on opposite complementary DNA strands leads to DNA double-strand breaks and MN formation.^[8] One of the mechanisms that may lead to MN from chromosome loss events is hypomethylation of cytosine in centromeric and pericentromeric repeat sequences such as classical satellite repeats at pericentromeric regions and higher order repeats of satellite DNA in centromeric DNA. Pericentromeric heterochromatin of chromosomes 1, 9 and 16 is greatly elongated when cytosine is hypomethylated leading to either malsegregation of these chromosomes and/or their loss as MN probably due to inappropriate kinetochore assembly.^[9] Assembly of kinetochore proteins such as CENPA and CENPB at centromeres is generally affected by methylation status of cytosine as well as methylation of histones in which a reduction in heterochromatin integrity might interfere both with microtubule attachment to chromosomes and with the proper sensing of tension from correct microtubule-kinetochore connections. It is probable that mutations leading to defects in kinetochore and microtubule interaction dynamics could also be a cause of MN formation due to chromosome loss at anaphase.^[10]

Micronuclei (MN) formation is been significantly higher in Polycystic ovarian syndrome than healthy female volunteers and positively correlated with body mass index (BMI), LH and Testosterone.^[11] The role of age as a confounding factor in cytogenetic biomonitoring found in age related increase of MN frequency where as contradictory results were reported in chromosomal aberration (CA) and sister chromatid exchange (SCE).^[12] There is increase Chromosomal damage with increase in Age leads to increase micronuclei frequency.^[13] In vitro exposure of peripheral lymphocytes to low dose gamma and neutron ionising radiation results in the formation of small chromosome fragments culminates to form micronuclei that may persist for the lifetime of the cell. An increased frequency of micronuclei in a cell may be considered as a biomarker of permanent genotoxic damage.^[14] Significant incidence of Micronuclei formation in pregnant women and cord blood samples after exposure of bombing in Serbia.^[15] MN frequency in PBL is a predictive biomarker of cancer risk within a population of healthy subjects. MN assay provides a valuable opportunity to apply this assay in the planning and validation of cancer surveillance and prevention programs.^[16] CBMN assay is established as biomarker of detecting Micronuclei (MN), Nucleoplasmic bridges (NPB), Nuclear buds (NB) in Lung cancer.^[17] Exposure to Xanthine, Hypoxanthine and super oxide dismutase (SOD) cause increase in the frequency of micronuclei.^[18]

MATERIALS AND METHODS

The materials required for the CBMN assay are the following viz. Reagents, Instruments and Consumables have been depicted in the Table-1 and the protocol for the preparation of stock and working solution of Cytochalasin-B, Hypotonic solution, Fixation, Sorens buffer and Giemsa Stain have been showing in Table-2.

Table 1: Showing Materials required for CBMN assay.

Sl. No	Materials	Specification	Company/Firm
1.	Cytochalasin-B	C6762 -1mg	SIGMA-Aldrich
2.	DMSO	RM 5856	HIMEDIA
3.	RPMI-1640	With glutamine without NaCo3	HIMEDIA
4.	Fetal Bovine Serum-10%	RM 111-500 ml	HIMEDIA
5.	Phytohaemagglutinin (PHA)	1%	SIGMA
6.	Pencillin-Streptomycin	5000 IU	SIGMA
7.	Gentamicin	Lot 124K2325	SIGMA
8.	Potassium Chloride AR	M.W 74.55	
9.	Methanol	Prod. No.32407	Fisher scientific
10.	Glacial Acetic acid	99-100%	MERC
11.	Centrifuge	Fixed Angle Rotator	REMI
12.	Incubator	12A 230 H VAC, 50/60 HZ	HICOOL
13.	Laminar Hood	Mod. No CAV 900	CLEANAIR
14.	Hot Air Oven	Auto clone	TECHNICO
15.	Vortex Mixer	230V/50 HZ	KEMI
16.	Slide Warmer		KEMI
17.	Deep freezer		VEST FROST
18.	Epifluorescent microscope-CCD Camera	BX-51	OLYMPUS
19.	Coplin jar		HIMEDIA
20.	Sterile hypodermic syringe	2ml	PRICON
21.	Needle	24G, 0.55x38mm	Oyster
22.	Disodium Orthophosphate	M.W-358.14	BURGOYNE
23.	Pottasium hydrogen phosphate	M.W-136.09	LOBA
24.	Potassium Chloride AR	M.W-74.55	LOBA
25.	Analytical Balance	Digital 0.1 mg-200 gm	Shimadzu
26.	Glass Beaker	500ml	
27.	Microscope Plain Glass Slide	75x25mm	
28.	Conical Flask	100,500 ml	
29.	Glass Marking Pencil		Faber Castil
30.	Slide Trey		
31.	Slide Storage Box		
32.	Culture vials		
33.	Centrifuge Tube	15ml	
34.	Pasteur Pipette	5.75 in	
35.	Micropipette	10-100µl	
36.	Transparent Staining Trough		
37.	Squeeze Bottle		
38.	Sterile hypodermic syringe	2ml	PRICON
39.	Needle	24G, 0.55x38mm	Oyster

Table 2: Showing Preparation of stock and working solution for Micronucleus test.

Sl. no	Reagent	Stock solution			Double distilled water	Working Solution
		Name of Chemical	Requirement	Mol.Wt		
1.	Cytokinesis inhibitor	1.Cytochalasin-B 2.DMSO	1 mg 0.4 ml	399.4 78.13	- -	-
2.	Hypotonic solution	(0.075M) KCl	0.559 gm	74.55	100 ml	-
3.	Fixatives	Methnol & Acetic acid	3:1	32.04 60.05	-	Based on requirement
4.	Sorensen's buffer	Na ₂ HPO ₄ M KH ₂ PO ₄	4.1740 gm 4.1620 gm	358.14 136.9	490 510	-
5.	Giemsa Stain	Geimsa Glycerine	3.8 gm 250 ml	291.80 92.09	-	48 ml Sorensen's Buffer +2 ml Giemsa stain

CBMN Assay is a versatile biomarker to detect DNA damage at Chromosome level in which its methodology has the following steps such as Preparation of Culture media, Inoculation of blood, Harvesting of Culture, Treatment with Hypotonic buffer, Treatment with Fixatives, Slide Preparation, Staining of slides, Microscopy - Scanning /Scoring By Manual methods and Analysis of data. This methodology was standardised during the doctoral research work of the first author on DNA Damaged Studies in Perinatal Asphyxia at Division of Cytogenetics in the department of Anatomy in collaboration with Division Neonatology and Biochemistry of departments of JIPMER-Pondicherry, India.^[21]

METHODOLOGY

Preparation of culture media

Culture media has to be prepared under aseptic conditions by following conventional standard as described by Rooney.^[5] 20ml stock solutions of RPMI - 1640 with folic acid was taken separately in sterilised beakers and 180 ml of double distilled water was added and made up to 200ml. Of which only 160 ml has to be taken and mixed with 40ml of fetal bovine serum, 05 ml of Phytohemagglutinin ,0.2ml of Penicillin-Streptomycin and 0.2 ml of Gentamycin have to be added to the above and were mixed thoroughly (Table:2). Culture media thus made has to be aliquoted into 5ml each in screw capped culture bottles. Then bottles have to be transferred into -20^oc refrigerator until further use.

Inoculation of blood - Setting up of culture

Required culture vials (Table:1) of each category stored in the deep freezer have to be taken out from the deep freezer and allowed to go for thawing under UV light exposure in laminar flow cabinet. 5-8 drops of heparinised peripheral blood drawn from cases and controls have to be added to culture vial. After gentle shaking, the bottles have to be screw capped under aseptic conditions (after showing to flame of sprit lamp). The culture vials were appropriately labelled and transferred to incubator, stabilised to 37^o C for 72hours.

Harvesting of culture

Between 44th hour of incubation, culture bottles have taken out from the incubator and placed in laminar flow cabinet. About 10 µl of working solution of **Cytochalasin- B** has to be added to the culture bottles under aseptic conditions (Table:2). The bottles were then transferred back to incubator and incubated for a period of 28 hours. At the end of 72nd hour of incubation, the contents of the bottle has to be transferred into 15ml centrifuge tubes and centrifuged at 1000 rpm for 10 min. After centrifugation, supernatant has carefully aspirated using suction apparatus without disturbing the cell sediments in the bottom of the tubes(Figure.1:6,7&8).

Treatment with Hypotonic solution

Placing the centrifuge tube over a Cyclomixer, 10ml of pre-warmed (37 °C) KCl hypotonic solution has to be added to the cell sediments, dispersed uniformly and kept for 1 minute at room temperature. Following KCl treatment, test tubes have taken out and centrifuged at 700-800 rpm for 10 min and the supernatant has to be discarded once again using suction apparatus (Figure.1:9).

Treatment with Fixative

The cells have to be re-suspended using freshly prepared fixative and kept 30 minute in refrigerator (Table:2). After 30 minutes the test tubes have to be taken from the refrigerator and brought to room temperature for re-centrifugation at 700-800 rpm for 10 mins and for a further wash with fixative. The same procedure has to be repeated thrice or until clear visible cell pellet collection / formation at the bottom of the test tube was achieved (Figure.1:10,11&12).

Preparation of slides

Cell pellets obtained after fixation alone has to be aspirated using the pasteur pipette from the bottom of test tubes and dropped onto a clean cold slide from a height of about 5-7 cm. For the purpose of identity of details of the cases, number codes has to be marked using glass marking pencil on the slides. Then they have immediately keep on a slide warmer maintained at 50-55°C until drying and later transferred and stored in slide racks. Slides were then subjected for staining after a

period of 24-48 hours after the preparation of slides (Figure.1:13).

Staining by Giemsa

Giemsa (2ml working solution +48ml Sorenson's buffer) and tap water have taken in clean, grease free Couplin jars arranged separately (Table:2). Later the slides were placed in Giemsa stain for 3 minutes followed by a rinse in tap water. The slides have to be kept over the slide warmer for drying(Figure1:14).

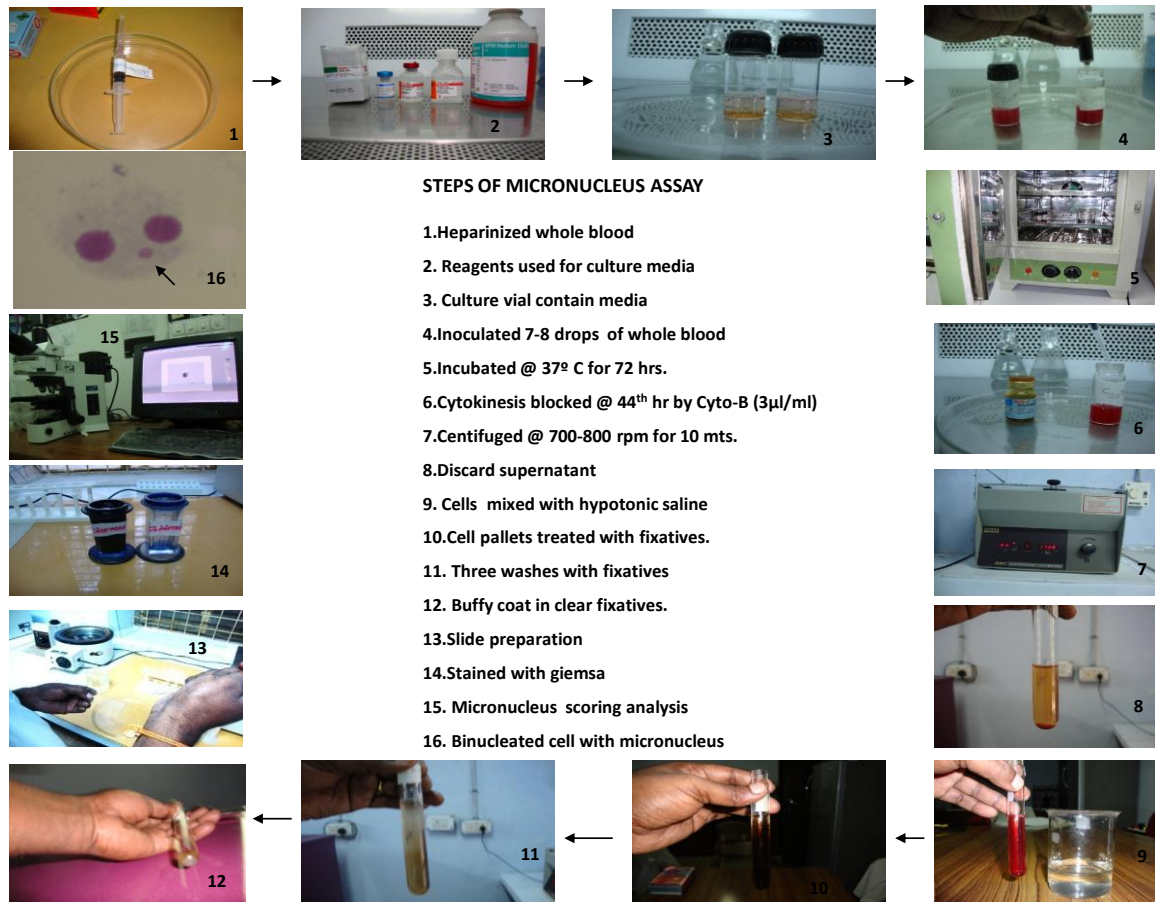
Microscopy –Scanning/ Scoring by manual method

The stained slides have to be examined under the microscope with bright field microscope BX 51 (Olympus) .The slides were scanned using low power 10 X. Then binucleated cells were scored blindly. The number of micronuclei per 1000 binucleated cells were captured and recorded .The following are the criteria to be followed for micronucleus scoring (Figure1:15&16).

1. The prerequisite of the micronucleus assay is preservation of cytoplasm of the binucleated cells.
2. The two nuclei of the binucleated cell must have similar size.
3. The two nuclei can touch each other or partially overlap.
4. The micronuclei should be morphologically identical but smaller than the nuclei.
5. The shape of MN is round or oval in appearance.
6. Diameter of the MN between 1/3rd and 1/16th of main nuclei.
7. Similar staining intensity as main nucleus.

Analysis of Data

The micronucleus index (MNi) for each sample has to be analysed by counting 1000 binucleated cells by manual method based on the scoring criteria framed by International collaborative project on micronucleus frequency HUMN project (2) (Figure.2).



STEPS OF MICRONUCLEUS ASSAY

- 1.Heparinized whole blood
2. Reagents used for culture media
3. Culture vial contain media
- 4.Inoculated 7-8 drops of whole blood
- 5.Incubated @ 37° C for 72 hrs.
- 6.Cytokinesis blocked @ 44th hr by Cyto-B (3µl/ml)
- 7.Centifuged @ 700-800 rpm for 10 mts.
- 8.Discard supernatant
9. Cells mixed with hypotonic saline
- 10.Cell pallets treated with fixatives.
11. Three washes with fixatives
12. Buffy coat in clear fixatives.
- 13.Slide preparation
- 14.Stained with giemsa
15. Micronucleus scoring analysis
16. Binucleated cell with micronucleus

Figure 1: Depicting Captures of Techniques of CBMN assay in clock wise direction from No.1 to 16 and its steps.

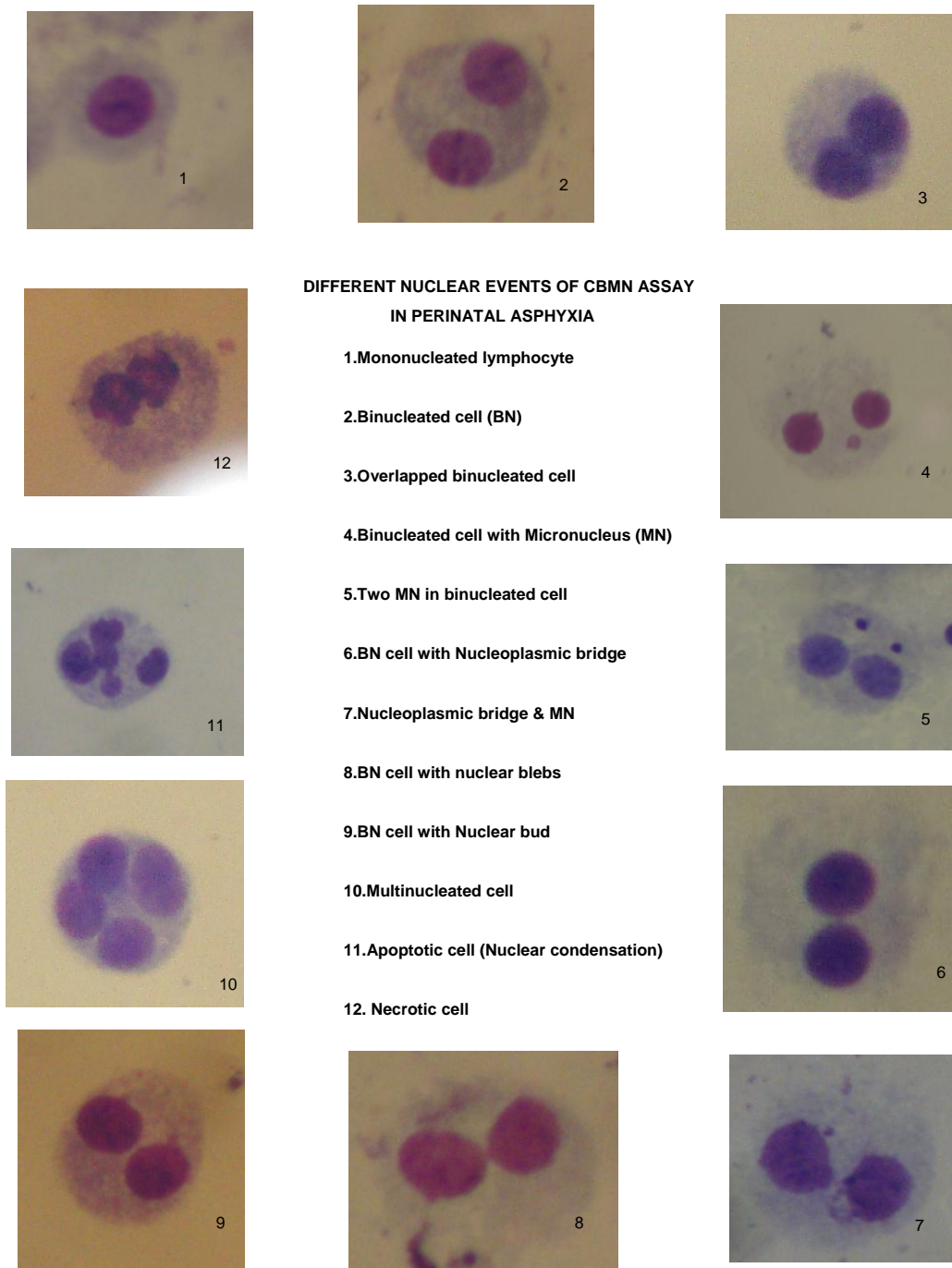


Figure 2: Depicting the Geimsa stained Captures of Various Nuclear Events of CBMN Assay in Clock-wise direction marked and stated with number 1 to12.

DISCUSSION

Cytokinesis Block Micronucleus (CBMN) Assay is being an established method to quantify genomic instability by blocking the cultivating lymphocytes at 44th hour after incubation by Cytochalasin-B to arrest the anaphase stage of cell division in order to detect double strand breakage and chromosome instability. The evidence of origin of micronucleus has been exemplified by the manifestation of un-repaired double strand break occurred in DNA which leads to formation of acentric

chromosome fragments and mal-segregation of chromosome during cell division depends upon the size of the micronuclei in a binucleated cell.^[2] The other nuclear anomalies observed in CBMN assay are Nucleoplasmic bridges (NPB) due to dicentric chromosomes in which the nucleoplasmic bridge connecting the two chromosomes in two poles. The nuclear budding (NBUD) has been characterised by appearance of mini circles connected to the nucleus by narrow stalk due to gene amplification during recombination between homologous regions.^[5]

Micronuclei formation and other nuclear anomalies like nucleoplasmic bridges (NPB) can be produced as the biproduct of dicentric chromosome which is the principal cause of genomic instability in one daughter cell. However lagging of dicentric chromosome or mis-segregation of one dicentric chromosome to one daughter cell can produce whole chromosome aneuploidy. The micronuclei frequency has been directly proportional to age which is more in female due to inactivation of X chromosomes in 72% and 37% is been due to kinetochore defects.^[6,7,8] Bloganessi et al in their studies proved that micronuclei formation has been increases when age advances.^[15,16] The defects in Genes BRCA1 and BRCA2 results unrepaired enzymes synthesis in homologous regions results in irreversibility in the DNA repair pathways due to molecular level instability of genomes.^[9] The incorporation of inappropriate base sequence of Uracil has been leading to double strand breaks which culminates in the formation of acentric fragments and micronuclei. Hypomethylation of Cytosine in centromeric and paracentric repeat sequence in chromosomes 1, 9 and 16 also leads to mal-segregation of chromosomes and inappropriate kinetochore resulting the origin of micronuclei.^[10,11] Methylation of histones of heterochromatin leads to interference in CENPA and CENPB proteins of kinetochore causes mutation in microtubule interaction dynamics results chromosome loss.^[12] The aforesaid surveys substantiating the molecular mechanism of geneomic instability for the origin of micronuclei in peripheral blood lymphocytes.^[2,3,6,7,8,9,10,11,12] E Yesilada et al conducted study in polycystic ovarian syndrome in which origin of micronuclei had been significantly increases compared to healthy women volunteers and positively correlated with BMI, LH and Testosterone.^[13] Exposure of PBL to low dose of gamma and neutron radiation causes chromosome fragments.^[16] Incidence of micronuclei frequency in cord blood samples of pregnant women before and after exposure to bombing in Siberia.^[17] Origin of Micronuclei in PBL of cancer patients is a predictive biomarker compared to healthy people.^[18] CBMN assay is one of the best biomarker for detecting Micronuclei index, Nucleoplasmic bridges, Nuclear buds in lung cancer.^[19] Reactive Oxygen Species (ROS) such as Super oxide dismutase, Xanthine, Hypoxanthine attacks nucleic acids causes adduct in DNA, if not repaired leads to DNA and Chromosome instabilities.^[20] Our study agrees with previous reports in which genomic instabilities have been due to disturbance of nucleic acid by invasion of free radical scavengers which leads to adduct in the single strands of DNA for it not been repaired resulting double strand breaks and acentric fragments leads the origin of Micronuclei and other nuclear anomalies compared to healthy controls for which micronuclei index was significantly increases in severity of Perinatal Asphyxia and positively correlated with Oxidative stress and Multiorgan dysfunction^[21,22] (Figure.2). By analysing the above reports CBMN assay proves that it is one of the best method for evaluation of Genotoxicity and Genomic instabilities.

CONCLUSION

CBMN Assay is reliable biomarker in order to quantify the Double strand break in DNA and Chromosomal instability in Peripheral blood lymphocytes which can be further evaluated with cytogenetic analysis whether the biproducts are being morphologically determined and strengthen their correlations.

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