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Nuclear and Cytoplasmic Abnormalities in the Fish *Catla catla* (Hamilton) Exposed to Chemicals and Ionizing Radiation

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ABSTRACT

The piscine erythrocyte micronucleus assay is a proven and sensitive indicator of DNA damage. Recently, several nuclear and cytoplasmic abnormalities other than Micronuclei (MN) have drawn wide attention. These include Nuclear Bud (NBu), Lobed Nucleus (LN), Nuclear Bridge (NBr), Anisochromasia (AN), Vacuolated Cytoplasm (VC) and Vacuolated Nucleus (VN). The importance of these nuclear abnormalities as biomarkers of exposures has not been previously exploited. In the present study an effort was made to compare the occurrence and frequencies of these nuclear abnormalities in fish exposed to sublethal concentrations of the insecticide Monocrotophos (MCP), herbicide butachlor and ionizing radiation in an effort to identify biomarkers specific to ionizing radiation and to validate these biomarkers for use in genetic toxicology studies. Among the various abnormalities, the occurrence of NBr was observed only in fishes exposed to ionizing radiation. The frequencies of NBu were significantly elevated in fish exposed to radiation. These observations indicate that NBr and elevated frequencies of NBu are likely indicators of radiation genotoxicity. The authors propose the erythrocyte MN assay to be more aptly renamed the 'erythrocyte micronucleus cytome assay' (ECMNA) considering the prospects of extending its applications in aquatic cyto-genotoxicity.

Key words: Pesticides, ionizing radiation, fish, erythrocyte micronucleus cytome assay

INTRODUCTION

Human population growth and rapid industrial development has caused an increase in the production, consumption and disposal of various chemicals and industrial wastes into seas, oceans and inland water bodies with potential accumulation of genotoxic and carcinogenic compounds. Fish are excellent organisms for the study of the mutagenic and carcinogenic potential of contaminants in the aquatic environment because they can metabolize, concentrate and store water borne pollutants (Al-Sabti, 1991). Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen xenobiotics that are potentially toxic. Moreover, fish genotoxicity is a matter of great concern because, apart from the potential to compromise the fishing industry and aquatic ecosystems there is a risk of exposure in organisms that consume these fish further up the food chain (Powers, 1989; Talapatra and Banerjee, 2007).

While the effect of toxic pollutants in large quantities will cause mortality in aquatic organisms, chronic low doses can induce endocrine disruptions and mutations triggering effects such as sex

reversals and neoplasia (Andersen *et al.*, 2002). Chemical agents like pesticides reach the aquatic ecosystem through direct applications, spray drift, spraying, washing from the atmospheric precipitation and run off from agricultural lands. Moreover, with increasing use of nuclear energy to meet the growing demands of developing nations, there is a need to monitor the effects of low concentrations of radionuclide releases in the environment. Radionuclides released from nuclear fuel cycles can become incorporated in the biogeochemical cycles of freshwater systems, having entered these systems in direct liquid discharges and/or through secondary processes such as erosion, runoff and groundwater infiltration from landscapes. As water bodies in the vicinity of nuclear establishments and repositories are likely to be contaminated with agricultural and industrial run-offs, identifying a biomarker specific to the effects of ionizing radiation would be an advantage to assess and monitor probable unintentional radionuclide leaks.

The International Commission on Radiological Protection (ICRP) has recently emphasized the need to protect non-human biota from the potential effects of ionizing radiation (ICRP-60, 1991; ICRP, 2001) and systematic studies using sensitive biomarkers are required to assess doses that can cause significant DNA damage in representative non-human species where sufficient data is not available. The genotoxic effects of ionizing radiation and other chemical toxicants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays but the erythrocyte micronucleus test has gained popularity over other basic cytogenetic assays in aquatic toxicity research due to its sensitivity, simplicity and reliability for detecting cytogenetic DNA damage and the short time needed to complete a study (Al-Sabti and Metcalfe, 1995; Cavas and Konen, 2007; Bopp *et al.*, 2008; Ali *et al.*, 2008).

Micronuclei (MN) are formed by chromosome fragments or whole chromosomes that lag at anaphase during nuclear division due to (1) the lack of a centromere in the case of acentric chromosome fragments or (2) dysfunctional centromeres or kinetochores or spindle in the case of chromosome loss (Heddle *et al.*, 1991). The frequency of micronuclei and other nuclear aberrations may thus provide evidence of the frequency of chromosomal damage and an insight into the risk of human health arising from presence of genotoxic environmental contaminants in aquatic ecosystems. In recent years, apart from MN formation, the formation of other cytoplasmic and nuclear alterations in piscine erythrocytes has been used as possible indicators of genotoxicity (Cavas and Ergene-Gozukara, 2005a, b; Da Silva Souza and Fontanetti, 2006; Ergene *et al.*, 2007; Cavas and Konen, 2008). Most studies on the erythrocyte MN assay have concentrated in the detection of MN frequency giving little importance to the various other nuclear abnormalities that can be easily scored as well. In the present study attempts were made to (1) Compare the various types of cellular alterations induced by gamma radiation and agrochemicals such as monocrotophos (organophosphate insecticide) and butachlor (chloroacetamide herbicide) in the erythrocytes of the freshwater fish, *Catla catla* in order to identify potential biomarkers of ionizing radiation (2) Validate a more comprehensive application of the erythrocyte MN assay to include various types of other nuclear abnormalities and consequently rename it as the Erythrocyte Micronucleus cytome assay (ECMNA). This approach is analogous to that already established for the cytokinesis-block micronucleus cytome assay in lymphocytes and the buccal cell micronucleus cytome assay (Fenech, 2007; Thomas *et al.*, 2009).

MATERIALS AND METHODS

Experimental fish specimens: Freshwater fish *Catla catla* (Hamilton, Family: Cyprinidae) was chosen for the study, as it is commonly available in India throughout the year and is an edible

species of considerable economic importance and also been proved to be a sensitive indicator of environmental stress (Tilak *et al.*, 1981). Fingerlings weighing between 8-10 g and of length 8 ± 2.0 cm were procured from a commercial fish farm and transported to the laboratory in oxygenated bags and released into 50 L glass aquaria filled with dechlorinated tap water. The specimens were given prophylactic treatment by bathing them twice in 0.05% potassium permanganate (KMnO_4) solution for 2 min to avoid any dermal infections. They were then acclimatized for 21 days under laboratory conditions with natural photoperiod and fed with rice-bran oil cake. The fecal matter and other waste materials were siphoned off daily to reduce ammonia content in water. The water quality parameters were analyzed and maintained within the normal range (pH-6.8, DO-8.2 mg L⁻¹, Temperature $-25\pm 1^\circ\text{C}$ and hardness-in terms of CaCO_3 was 220 ppm, respectively).

Exposure to radiation: Fish from the acclimatized set were placed in glass beakers (3 in a beaker) of one litre capacity containing tap water. The beakers were placed at a radial distance of 16 cm from a sealed Cs-137 gamma ray source (1.15 Ci, BRIT, Mumbai, India) and irradiated for 42 h at a dose rate of 2 mGy min⁻¹ to a total dose of 5 Gy. The irradiation set-up could accommodate about 10 beakers in a circle to provide uniform irradiation. The size of the glass beakers was kept small to ensure that all fish were given approximately the same radiation dose. After irradiation the fish were transferred to glass aquaria of 50 L capacity containing dechlorinated tap water. Fish from the same lot were sham exposed and used as controls. Blood was collected 72 h post exposure by severing the caudal peduncle and immediately processed for micronucleus assay. Mortality was not observed throughout the study. The treatment was carried out with five fish for each experiment and repeated four times for reproducibility of the results.

Determination of Sub lethal concentrations: The acute toxicity bioassays to determine the LC_{50} -96 h value of monocrotophos technical and butachlor 50% EC (emulsifiable concentrate) were conducted in the semi-static system using the arithmetic method of Karber adapted by Dede and Kaglo (2001). Stock solutions of MCP and butachlor 50% EC were prepared by dissolving in acetone and water, respectively. A set of 10 acclimatized *Catla catla* fish specimens was randomly exposed to five different concentrations of MCP (4.5, 6.8, 10, 15 and 23 ppm) and butachlor 50% EC (0.05, 0.1, 0.2, 0.4 and 0.8 ppm). The LC_{50} -96 h values of MCP and butachlor 50% EC were determined as 12.6 and 0.315 ppm, respectively.

Exposure to MCP: Technical-grade monocrotophos (36% SL) with trade name Parryfos (MCP; CAS No. 6923-22-4) (3-dimethoxyphosphinoyloxy-N-methylisocrotonamide) was purchased from Coramandel fertilizers Ltd. Secunderabad. MCP an organophosphate insecticide used in agriculture to control a variety of pests in paddy fields is still used in India. A total of five fish from the acclimatized set were exposed to a sublethal concentration of 2.5 ppm with respect to LC_{50} value and the experiment was repeated twice. The concentration of acetone was maintained 0.1% in test solution and solvent control. Mortality was not observed throughout the study. Blood was collected 72 h post exposure by severing the caudal peduncle and immediately processed for micronucleus assay.

Exposure to butachlor: Butachlor (50% EC), a widely used herbicide with trade name Weedar (CAS No. 23184-66-9) [N-(butoxymethyl)-2-chloro-2', 6'-diethylacetanilide] was purchased from

Sree Ramcides chemicals Ltd. Tamilnadu. A total of five fish from the acclimatized set were exposed to a sublethal concentration of 0.063 ppm with respect to LC_{50} value and the experiment was repeated twice. Mortality was not observed throughout the study. Fish maintained in tap water (sham exposed) were treated as control. Blood was collected 72 h post exposure by severing the caudal peduncle and immediately processed for micronucleus assay.

MN assay: Blood smears were made onto grease-free pre-cleaned slides. The MN assay was performed as per the protocol of Das and Nanda (1986) but with slight modifications. After fixation in pure ethanol for 10 min, the slides were allowed to air-dry and stained with May-Grunwald (Merck, Mumbai) solution for 15 min followed by 10% Giemsa for 30 min. Slides in duplicate were made for each fish and 3000 cells were scored under a light microscope (Nikon PFX Japan, Optiphot-2, Oil immersion lens, 100/1.25). The established criteria for identifying micronuclei were strictly followed to ensure authentic scoring (Huber *et al.*, 1983; Titenko-Holland *et al.*, 1997; Matsumoto and Colus, 2000). Cells having an oval appearance with intact cytoplasm, oval nuclei with an intact nuclear membrane, micronuclei less than or equal to 1/3rd the size of the main nuclei, micronuclei clearly separated from the main nuclei and micronuclei that were not refringent were considered for scoring.

Classification of cellular abnormalities: Cytological abnormalities observed in the present study were classified into two groups, Nuclear (NA) and Cytoplasmic Abnormalities (CAs). NAs include, cells having main nuclei with a clearly separated smaller nucleus termed a Micronucleus (MN); nuclei with a substantial indentation (notch) into the nucleus were noted as Deformed Nucleus (DN); nuclei containing euchromatin and having a relatively small evagination (bud) of the nuclear membrane as Nuclear Bud (NBu); Lobed Nuclei (LN) had evaginations larger than those in the nuclei, including those with several lobes. A bridge-like formation between two erythrocytes was identified as Nuclear Bridge (NBr). Nuclei containing vacuoles were termed as Vacuolated Nucleus (VN). Cells with two nuclei and a common cytoplasm were considered as Binucleated Cells (BNC). The CAs include anisochromatic erythrocytes with pigmented periphery and a virtually colorless central region (AN); whereas cytoplasm with vacuoles was termed Vacuolated Cytoplasm (VC). Cells with spine like projections at the periphery of the cytoplasm were considered as Echinocytes (EC) and erythrocyte bearing cytoplasm alone as Eucleus (EN). Erythrocytes that are miniature in size were considered as Microcyte (MC).

Statistical analysis: Statistical analyses were performed using the INSTAT software. All data from the assays were tested for normality. Since the test did not show normal distribution, the non-parametric Welch's test was used. Statistically significant differences were tested at 1 and 5% levels.

RESULTS

After exposure to gamma radiation or MCP or Butachlor, the peripheral erythrocytes were analyzed for the presence of various abnormalities. Apart from the induction of micronuclei, other abnormalities (depicted in Fig. 1) were observed. Details of the various types of abnormalities and their frequencies are given in Table 1. The column indicated as solvent control refers to samples sham exposed with respect to MCP but including the solvent vehicle, acetone. In the present study, a protracted dose of 5 Gy at a dose rate of 2 mGy min^{-1} was compared with sub-lethal doses of MCP and butachlor. The results were analyzed on day 3 after single exposure. The various cellular

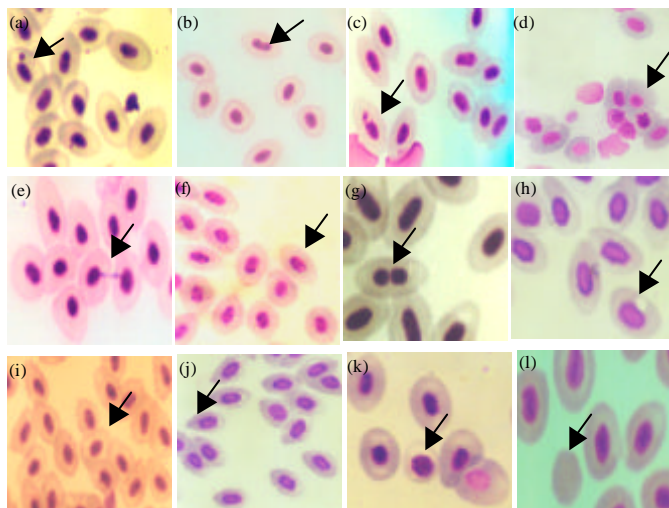


Fig. (1a-l): Representative images of various erythrocytic cellular abnormalities in the fish *Catla catla* exposed to chemicals and ionizing radiation; (A) Micronucleus (MN); (B) Deformed nucleus (DN); (C) Nuclear bud (NBu); (D) Lobed nucleus (LN); (E) Nuclear bridge (NBr); (F) Vacuolated nucleus (VN); (G) Binucleated cell (BNC); (H) Vacuolated cytoplasm (VC); (I) Anisochromasia (AN); (J) Echinocyte (EC); (K) Microcyte (MC); (L) Enucleus (EN)

Table 1: Frequencies of various erythrocyte abnormalities in the fish *Catla catla* exposed to chemicals and ionizing radiation

Types of aberrations	Gamma Radiation		Butachlor		Monocrotophos	
	Control Mean±SE	(5 Gy) Mean±SE	Control Mean±SE	(0.063 ppm) Mean±SE	Solvent control Mean±SE	(2.5 ppm) Mean±SE
MN	0.3±0.20	2.95±0.28*	0.2±0.12	0.55±0.13	0.3±0.20	0.9±0.12
DN	1.0±0.16	30.42±5.53*	0.4±0.18	8.4±0.38*	0.8±0.37	2.15±0.24
NBu	0.4±0.30	12.60±1.61*	0.5±0.22	4.15±0.21	0.7±0.25	4.9±0.38
LN	-	1.85±0.74	-	0.3±0.15	-	2.0±0.16*
NBr	-	0.27±0.11	-	-	-	-
VN	0.5±0.22	2.95±3.11	0.3±0.12	0.8±0.13	0.3±0.20	1.65±0.23**
BNC	-	-	-	0.5±0.18	-	-
VC	0.8±0.25	17.0±4.02*	0.2±0.12	28.5±0.81*	0.4±0.18	1.85±0.15
AN	0.4±0.18	2.27±0.40*	0.2±0.12	8.55±0.39*	0.2±0.12	2.9±0.99
EC	-	-	-	0.35±0.16	-	1.05±0.40
MC	-	-	0.2±0.12	0.2±0.11	0.2±0.20	0.25±0.13
EN	-	1.62±0.64**	-	0.1±0.06	-	-

*Statistically significant at 1% level, ** Statistically significant at 5% level, Note: MN: Micronuclei; DN: Deformed nucleus; NBu: Nuclear bud; LN: Lobed nucleus; NBr: Nuclear bridge; VN: Vacuolated nucleus; BNC: Binucleated cell; VC: Vacuolated cytoplasm; AN: Anisochromasia; EC: Echinocytes; MC: Microcyte; EN: Enucleus

alterations observed in the present study were MN, DN, NBu, LN, NBE, VN, BNC, VC, AN, EC, MC and EN. When compared with control and solvent control, significant increases in the frequency of cellular abnormalities were observed in fish exposed to gamma radiation, MCP and butachlor, respectively.

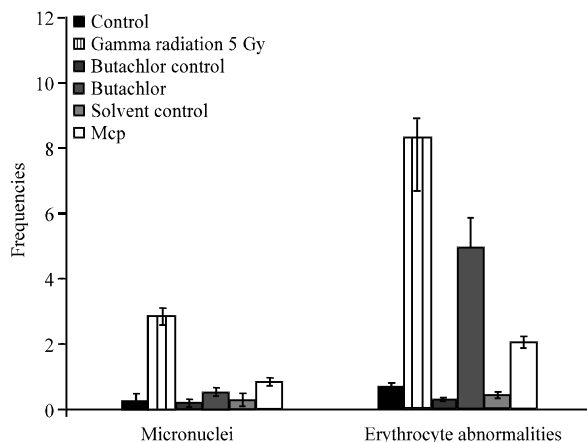


Fig. 2: Frequency of MN and total erythrocyte abnormalities in peripheral blood of *Catla catla* exposed to chemicals and ionizing radiation

MCP was found to induce a variety of cellular alterations at a concentration of 2.5 ppm. Statistically significant increase in the frequency of LN and VN were noted when compared to butachlor exposure. A significant increase in the frequency of DN, VC and AN were observed after exposure to butachlor. Binucleated cells were noted only in butachlor exposed fish. Compared to gamma radiation or MCP, fish treated with butachlor showed an elevated frequency of VC and AN.

An increase in MN frequency was observed in fish irradiated with gamma radiation. Statistically significant increase in the frequency of DN, NBu and EN were observed in irradiated fish compared to those exposed to MCP or butachlor. It may be noted that compared to MCP or butachlor exposure, a 3 fold increase in the frequency of nuclear bud occurred in fish exposed to gamma radiation.

The frequencies of total erythrocyte abnormalities (other than MN) in comparison to MN are shown in Fig. 2. Statistically significant increase in the erythrocyte abnormalities were observed in treatment groups when compared to the control ones. Among these, the abnormalities induced by gamma radiation are significantly greater than butachlor and MCP. Compared to the frequency of micronuclei, other abnormalities put together account to a significant number.

DISCUSSION

The erythrocyte micronucleus test is a preferred bioindicator of environmental mutagenicity. During micronuclei analyses, some authors have observed the occurrence of other nuclear abnormalities, suggesting that they must also be taken into consideration as potential indicators of cytotoxicity (Schroeder, 1970; Tolbert *et al.*, 1991, 1992; Fenech *et al.*, 1999). In fish, these nuclear alterations have been reported after exposure to chemical agents or polluted waters (Cavas and Ergene-Gozukara, 2003; Da Silva Souza and Fontanetti, 2006).

The effects of low dose and dose-rate ionizing radiation on fish are lacking and those available are mainly the effects on the reproductive system (Woodhead, 1977; Hyodo-Taguchi, 1980; Shima and Shimada, 1991). A few cytogenetic studies show significant aberrations induced by ionizing radiation. Mong and Berra (1979) reported a dose-dependent increase in aberrant metaphases such as chromatid breaks, gaps and chromatid interchanges in fish irradiated

with 3.3-9.4Gy of X-rays. Suyamaa and Etoh (1983) X-irradiated lymphocytes from mud minnows with 0.48-1.9Gy and scored dicentrics with acentric fragments. The erythrocyte MN assay is easier to perform as it does not require culturing and it includes detection of DNA damage in addition to other cellular changes. However, most studies focus on the induction of MN with little importance to other cellular abnormalities (Manna *et al.*, 1985; Kurihara *et al.*, 1992; Schultz *et al.*, 1993; Bahari *et al.*, 1994; Gustavino *et al.*, 2001; Takai *et al.*, 2004; Cassidy *et al.*, 2007). Nevertheless, the presence of cellular abnormalities, besides micronuclei, has been reported by several authors in humans (Schroeder, 1970; Tolbert *et al.*, 1991; Titenko-Holland *et al.*, 1997; Hamza and Mohankumar, 2009) and fish cells (Ayllon and Garcia-Vazquez, 2000; Ateeq *et al.*, 2002; Pacheco and Santos, 2002; Cavas and Ergene-Gozukara, 2003; Da Silva Souza and Fontanetti, 2006; Ergene *et al.*, 2007). In the present study along with MN, various cytoplasmic and nuclear abnormalities were observed in fish exposed to low doses of MCP or gamma radiation.

In the present investigation a 72 h exposure period was selected taking into consideration an estimated time of 1-3 days after a clastogenic treatment for the detection of MN in circulating blood erythrocytes as in studies conducted by Cavalcante *et al.* (2008) and Nwani *et al.* (2010) with pesticides and Takai *et al.* (2004) in the fish *Oryzias latipes* 24 h after exposure to X-rays. Apart from MN, deformed nucleus (notched nuclei), nuclear bud (blebbed nucleus), binucleated cells, lobed nucleus, vacuoles in cytoplasm and nucleus were observed by many investigators in fish exposed to various genotoxicants (Ayllon and Garcia-Vazquez, 2000; Cavas and Ergene-Gozukara, 2003; Cavas and Ergene-Gozukara, 2005a, b; Da Silva Souza and Fontanetti, 2006; Ergene *et al.*, 2007). Erythrocytic nuclear abnormalities have been used by many as a signal of cytogenetic damage in fish species (Metcalf, 1988; Pacheco and Santos, 1997).

In the present study, a butachlor concentration of 0.064 ppm was found to induce a 10 fold increase in VC compared to gamma and a 20 fold increase compared to MCP. Exploring for biomarkers, Ateeq *et al.* (2002) observed EN only in fish exposed to butachlor thereby proposing EN a specific biomarker of butachlor exposure. However, our study indicates that ionizing radiation can also induce EN. In the present study the formation of binucleated cell is observed only in fish exposed to butachlor and not in others. This may be attributed to the blocking protein synthesis and thereby inhibition of cell division (Panneerselvam *et al.*, 1999).

Monocrotophos belongs to the phosphorous ester group and is capable of inducing mutation by virtue of its alkylating activity (Eto and Ohkawa, 1970). The property of alkylation leads to DNA damage that result in the formation of micronuclei and other nuclear abnormalities observed in the present study.

Ionizing radiation is known to induce DNA double strand breaks causing the formation of dicentric chromosomes that are manifested as nucleoplasmic bridges at cytokinesis (Fenech *et al.*, 2011). In the present study, bridges between two erythrocytes were observed along with the aforesaid nuclear alterations albeit at a low frequency. It may be speculated that the elevated frequency of nuclear bud and lobed nuclei observed in the present study are in fact broken bridges. Although a few reports indicate nuclear buds as precursors of MN (Shimizu *et al.*, 1998, 2000) it may be mentioned here that in an extended version of the present study in which these cellular abnormalities were monitored 45 days post exposure, no significant increase in MN with proportional decrease of NBu and LN was observed in subsequent days after the 72 h post irradiation period. Hence, the nuclear buds and lobed nuclei observed by us are likely to be the resultants of nuclear bridges. This hypothesis is also supported by the observations of "tailed nuclei" in fish exposed to ionizing radiation by Prokofjeva-Belgovskaya (Prokofjeva-

Belgovskaya, 1961). The formation of such tailed nuclei was later verified in non-dividing human lymphocytes from Chernobyl liquidators (Kravtsov *et al.*, 2000). Considering the fact that nucleoplasmic bridges have proved to be sensitive indicators of ionizing radiation in human blood lymphocytes (Khan *et al.*, 1994; Thomas *et al.*, 2003; Hamza and Mohankumar, 2009) the resultants of broken bridges could also serve as biomarkers of exposures to ionizing radiation in fish erythrocytes.

To our knowledge, this is the first study devoted to the comparison of cellular alterations in fish exposed to ionizing radiation and pesticides in order to identify a biomarker of radiation. Although this preliminary investigation points to certain biomarkers of ionizing radiation, elaborate studies with various doses and dose-rates are required before these abnormalities find application as prospective biomarkers in aquatic radiation biodosimetry. The study also suggests that the erythrocyte MN assay can be aptly renamed the "Erythrocyte micronucleus cytome assay" (ECMNA) as it encompasses a variety of biomarkers that may find application in genotoxicity. It may be noted that due to the various additional information the other abnormalities provide Fenech (2007) has now termed the human lymphocyte micronucleus assay as the cytokinesis-block micronucleus cytome assay.

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