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In vitro micronucleus test assessment of polycyclic aromatic hydrocarbons*

Kamila Żelazna[1], Katarzyna Rudnicka[2], Sebastian Tejs[2]

[1] Department of Plant Breeding and Seed Production, University of Warmia and Mazury in Olsztyn, Łódzki 3, 10-957 Olsztyn, Poland, E-mail: kamila.zelazna@uwm.edu.pl
[2] Department of Environmental Biotechnology, University of Warmia and Mazury in Olsztyn, Słoneczna 45g, 10-712 Olsztyn, Poland

ABBREVIATIONS

BN – binucleated
CBMN – cytokinesis block micronucleus assay
Cyt-B – cytochalasin-B
FISH – fluorescent in situ hybridization
MN – micronucleus
MNi – micronuclei
NBUDs – nuclear buds
NPB – nucleoplasmic bridge
PAH – polycyclic aromatic hydrocarbon

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) can be dangerous due to their genotoxic properties and the formation of DNA adducts in the target organs. The increasing number of environmental pollutants containing polycyclic aromatic compounds requires the use of inexpensive and relatively quick assays enabling evaluation of potential genotoxicity of chemicals. Therefore, the *in vitro* micronucleus assay (MN) has been developed as a rapid screening test based on measuring and counting the micronuclei in various cells subjected to the examined compound. Combined with the cytokinesis-block micronucleus (CBMN) technique and the fluorescence *in situ* hybridization (FISH) assay, it also enables classification of compound mutagenic activity into aneugenic or clastogenic categories, the identification of the mechanism of MNi formation and the estimation of malsegregations of chromosomes amongst the two nuclei in binucleated cells. The combination of these different approaches is very useful in performing risk assessments of PAH with threshold types of dose responses.

INTRODUCTION

Many compound environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs), are not carcinogenic themselves. Their carcinogenic activity is generated on the metabolic pathway (Łuczyński et al. 2005). These organic compounds are metabolically activated to highly reactive PAH diol epoxide derivatives, which can covalently bind to native DNA to implement their mutagenic and carcinogenic activity (Wu et al. 2003). The metabolites of PAHs often induce DNA damage and repair synthesis or the formation of DNA adducts in the target organs of their carcinogenic activity, as well as in the primary cultures of cells (Ku et al. 2007). Moreover, PAHs are lipophilic chemicals which are subject to bioaccumulation through the food chain (Minissi et al. 1998), thereby increasing the risk of cancer. Polycyclic aromatic hydrocarbons are ubiquitously distributed in our environment as by-products of tobacco smoke, incomplete combustion of fossil fuels, the discharge of crude oil and petroleum products and various industrial processes (Rybacovas et al. 2007). These compounds are mainly of anthropogenic origin, but may also arise from non-anthropogenic sources, such as in soil as a result of the degradation of organic matter (Thiele and Brummer 2002). Many studies have been devoted to examining the known and

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unknown mutagenic or genotoxic properties of environmental pollutants. In the case of studying the properties of polycyclic aromatic hydrocarbons (PAH), they are usually considered to be a group (mixture) of various compounds occurring in the environment and, consequently, the results concern not one, but a mixture of several compounds. Depending on the source and the presence of different levels of environmental pollutants (air, water or soil), a battery of *in vivo* and *in vitro* genotoxicity tests are used.

This paper presents one such assay developed to study the aneugenic/clastogenic properties of polycyclic aromatic hydrocarbons. In the case of single synthesized PAHs, with a known structure and purity, it is necessary to apply a simple and inexpensive pre-screening method. Initially, one type of *in vitro* genotoxicity tests used to study properties of PAHs were the bacterial tests developed by Ames et al. (1975). The *Salmonella* Typhimurium/microsome assay (*Salmonella* test; Ames test) was specifically designed to detect chemically-induced mutagenesis (Ames et al. 1975). The *Salmonella* mutagenicity test is a widely accepted short-term bacterial assay for identifying substances which can produce genetic damage leading to gene mutations. This method is based on different point mutations in various genes in the histidine operon (Tejs 2008). This test is very well-suited for initial examination of the mutagenicity properties of PAHs. Since the point mutation test is sometimes not able to detect all genotoxic effects of PAHs and false-negative results were obtained (Ying et al. 2004), a better solution is to combine the Ames test with another *in vitro* test, which was developed as a method to measure structural chromosomal damage, namely, an *in vitro* micronucleus test (Lorge et al. 2006). This genotoxicity test detects compounds which induce genetic damage directly or indirectly by various mechanisms (Yılmaz et al. 2009). Genotoxicity is expressed as varying types of DNA damage and mutations, ranging from gene to structural or numerical chromosome changes (aneuploidy and polyploidy) (Mateuca et al. 2006). Therefore, the *in vitro* micronucleus test is an excellent tool to supplement knowledge of the genotoxic properties of PAHs.

A CORE *IN VITRO* MICRONUCLEUS TESTS

The genotoxic potential of environmental pollutants in water, air and soil can be investigated using various biomarkers and assay systems. Current genotoxicity testing guidelines incorporate a battery of *in vitro* and *in vivo* tests to identify hazards. Cytogenetic damage evaluation is an important step in a range of different approaches used in genetic toxicology. The micronucleus (MN) test is among the most sensitive DNA damage indicators and it has been applied to several organisms and tissues for the evaluation of environmental contaminants (Lemos et al. 2011). The ability of the micronucleus assay to detect both clastogenic and aneugenic effects (leading to structural and numerical chromosome alterations, respectively) is a key advantage of this methodology. The distinction between the two phenomena (by identifying the origin of micronuclei (MNi)) is important for whether micronucleus analysis is used for genotoxicity testing or for biomonitoring of genotoxic exposure and effects in various cells (Attia et al. 2009). This might appear important in cases where the ultimate toxic chemical entity may be a metabolite of the parent chemical agent such as polycyclic aromatic hydrocarbon. There are relatively new methods based on micronuclei (MNi) formations *in vitro* in dividing cell cultures which are based on these disturbances occurring during mitosis. This condition can be caused by organic chemicals whose targets include components of the cytoskeleton and chromosome condensation or the spindle apparatus (Dopp et al. 1995).

Micronucleus (MN) refers to the fragment of damaged chromosomes or whole chromosomes which fail to find their way onto the spindle during cell division. At anaphase, when the centric elements move towards the spindle pole, the centric chromatids and chromosomal fragments lag behind. After telophase, the undamaged chromosomal fragments as well as the centric fragments give rise to regular daughter nuclei. Although the lagging elements are also included in the daughter cells, a considerable portion are transferred onto one or several secondary nuclei, which are much smaller than the principle nuclei and are generally referred to as "micronucleus" (Gangar et al. 2010).

An *in vitro* MN assay can detect both clastogens and aneugens as well as mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction (Corvi et al. 2008). The MNi can result from whole chromosome loss or chromosome breakage events and chromosome lagging due to a dysfunction of the mitotic apparatus. The predominant induction of either type of micronucleus can be used to classify chemical activity into either aneugenic or clastogenic categories (Parry and Parry 2006).

Clastogenic agents cause chromosomal rearrangements and damage mainly through interaction with DNA to form acentric fragments of chromosomes (Saleh and Sarhan 2007). Chromatid or chromosome fragments and entire "aberrant" chromosomes which are not included in the newly-formed daughter cells form micronuclei (Figure 1). The MN assay is more rapid and simpler than chromosomal analysis (Gandhi et al. 2003). Compared to chromosome aberrations, the final result is relatively easily scored and thus requires less time to make an assessment of the clastogenicity of a chemical (Garriott et al. 2002).

Not only does the *in vitro* micronucleus test allow the detection of clastogens as genotoxicants, but it also enables their discrimination from aneugens (Lorge et al. 2006). The aneugenic agents induce chromosomal loss mainly through interference with the spindle apparatus (Saleh and Sarhan 2007). Yang and followers (2003) presented several possible paths of aneupliody which result in MNi (Figure 1). One of the common mechanisms for aneuploidy is mis-segregation of lagging chromosomes, or laggards. Laggards are displaced mitotic chromosomes that are frequently encapsulated in a micronucleus and excluded from the daughter nuclei as mitosis ends (Yang et al. 2003).

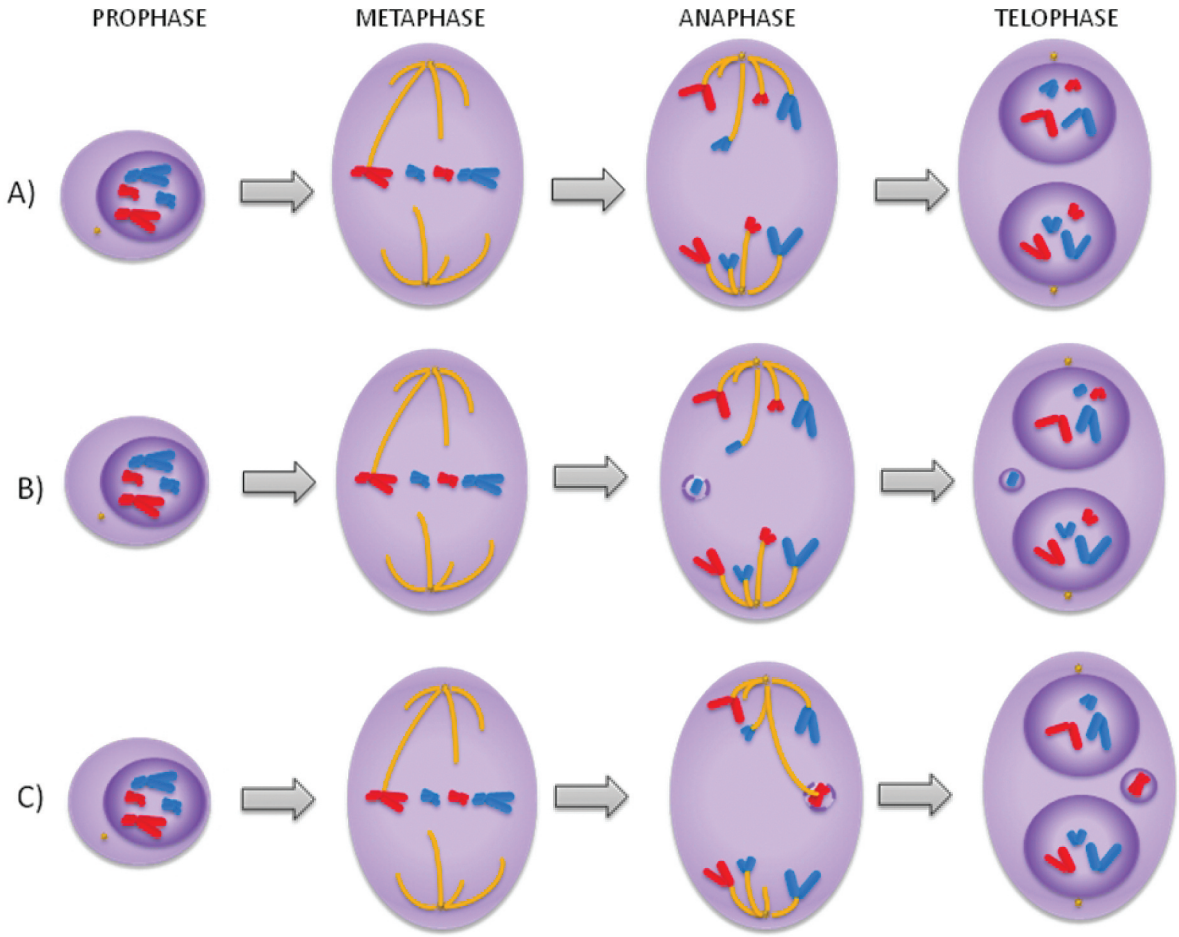

Figure 1. Different mechanisms leading to micronuclei formation. A) proper mitosis; B) clastogenic effect; C) aneugenic effect.

Although micronuclei constitute well-characterized biomarkers of chromosomal damage (Salazar et al. 2009), the in vitro MN test does not provide information on the origins of micronuclei (Corvi et al. 2008). A convincing demonstration of the presence of whole chromosomes or only chromosome fragments in micronuclei depends upon the availability and application of suitable technologies (Parry and Parry 2006), for example, fluorescence in situ hybridization. This method is a molecular cytogenetic technique which can be used to obtain information from metaphase or interphase cells, depending on the specific sequence of the fluorochrome conjugated probe applied. Using centromeric probes, the binding of the probe to its target can be identified by a distinct fluorescence signal along metaphase chromosomes and within cell nuclei. For visualization of the aneugenic or clastogenic effect of investigated compounds in micronuclei of cell line V79 of Chinese hamster lung fibroblasts, the fluorescent *in situ* hybridization method (FISH) will be used with centromere probes. FISH provides a convenient, rapid method for detection of the centromeric sequence in metaphase spreads and interphase nuclei.

THE USE OF CYTOCHALASIN-B ACTIVITY AND CYTOKINESIS-BLOCK IN MICRONUCLEUS TEST

The MN assay can only be effective as a quantitative biological dosimeter, provided one can identify those cells which have divided after exposure, since it is only dividing cells which can express micronuclei. This aim was achieved with the development of the cytokinesis-block micronucleus (CBMN) technique in the human lymphocytes (Fenech 1993). In the CBMN assay, cells that have completed one nuclear division are blocked from performing cytokinesis using cytochalasin-B (Cyt-B) and are consequently readily identified by their binucleated appearance (Fenech 2000).

Cyt-B is an inhibitor of actin polymerization required for the formation of the microfilament ring which constricts the cytoplasm between the daughter nuclei during cytokinesis, which prevents cytokinesis, but not nuclear division, resulting in cells with multiple nuclei (Figure 2). The use of this method enables the accumulation of virtually all dividing cells at the binucleate stage in dividing cell populations,

regardless of their degree of synchrony and the proportion of dividing cells. MNi are then scored in binucleated cells only, which enables reliable comparisons of chromosome damage between cell populations which may differ in their cell division kinetics. As a consequence, the CBMN assay appears to be more accurate and more sensitive than the conventional methods, which do not distinguish between dividing and non-dividing cells (Fenech 2007).

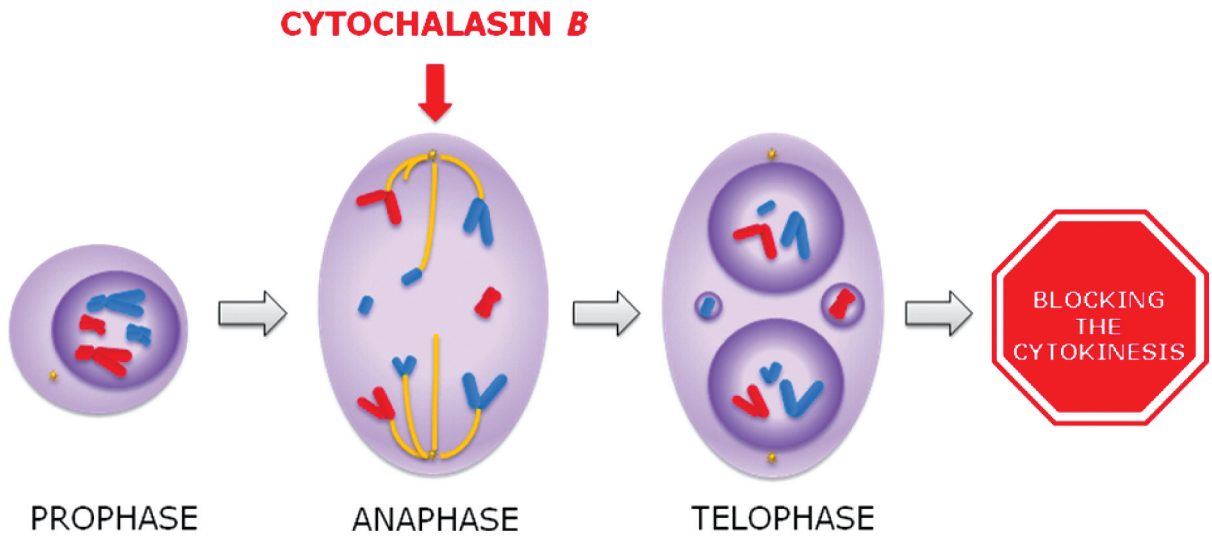

Figure 2. Dividing cell in CBMN assay. The role of cytochalasin-B in blocking dividing cells at the binucleated stage (based on Fenech 2007).

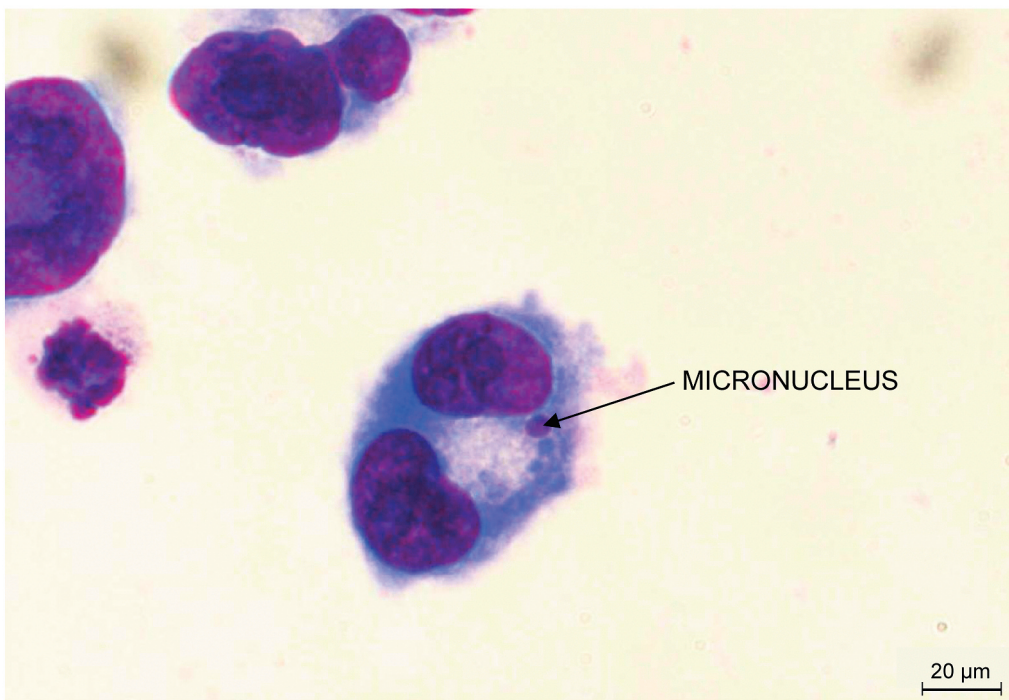

Figure 3. The binucleate V79 cell with micronucleus in the presence of cytochalasin-B (Rudnicka 2010).

The CBMN assay was proposed in 1985 by Fenech and Morley for human lymphocytes and since then it has been adapted for different cell types, relevant to human biomonitoring (Marzin 1997). The cytokinesis blocking method is also currently applied in the micronucleus test in the Chinese hamster established cell line. Other lines, such as V79 lung fibroblast and Chinese hamster ovary (CHO) (Matsushima et al. 1999), are also often used by researchers. These lines have the most known properties, e.g. length of cycle, adhesion and resistance for contagion. The result of this approach are binucleated (BN) cells and a micronucleus, which is the mutagenic response of the cell (Figure 3).

A variant with a cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MNi in cultured human and/or mammalian cells. The scoring is specifically restricted to once-divided cells. These cells are recognized by their binucleated (BN) appearance after inhibition of cytokinesis by cytochalasin-B. Restricting scoring of MN to BN cells prevents the distorting effects caused by suboptimal cell division kinetics, which constitutes a major variable in this assay (Fenech 2007).

A combination of these different approaches is very helpful in performing a risk assessment of polycyclic aromatic hydrocarbons with threshold-type dose responses.

MN ASSAY PROCEDURE TAILORED TO DETECTION OF GENOTOXIC PROPERTIES OF PAHS

All the procedures are based on ISO 21427-2, Water Quality - *Evaluation of Genotoxicity by Measurement of Induction of Micronuclei - Part 2: Mixed population method using the cell line V79*, with modifications from International Institute of Pharmacology in Warsaw. The methods and protocol are adapted for investigation of polycyclic aromatic hydrocarbons obtained from Jagiellonian University for non-commercial use.

Before beginning the micronucleus test, it is necessary to carry out a pre-incubation of the cell line, after at least four passages cells have the proper properties. As an initial step in the procedure, an MTT test for cytotoxicity has to be performed to establish the experimental concentrations of investigated chemicals. The procedure applied in the *in vitro* micronucleus test consists of four stages (Figure 4). All steps have the same conditions: 5% CO_2 atmosphere, a temperature of 37°C and 95% humidity. The test is carried out in two variants: with and without metabolic activation. To imitate the conditions observed in higher organisms, *in vitro* conditions require a similar co-factor which will transform chemicals. In this case, the metabolic fraction (S9 from rat liver) is used to detect if metabolites have a higher genotoxic impact than original chemicals. When beginning the *in vitro* test, cell lines are seeded on culture plates with a minimal essential medium and incubated for 24 hours. After the prime incubation, the medium is exchanged for a medium with examined concentrations of chemicals. This stage lasts 24 hours for the variant without the use of the metabolic fraction (S9), 1 hour for pre-incubation of tested chemicals with S9 fraction and 23 hours for cells with filtrate obtained from 1 hour pre-incubation. During the third stage, the medium is exchanged for a medium with $3\mu g \cdot ml^{-1}$ of cytochalasin-B in order to stop division of cells. At the end of the test, microscopic sections are preserved and dyed with the use of the Giemsa staining method (cytoplasm turns light blue and the nucleus violet-pink) when observed under a light microscope.

The result of the test is the percentage of binucleated cells with micronuclei-to-binucleated cells without micronuclei (in a pool of 1000 cells). The culture conditions are also taken into consideration. If cells have numerous cytoplasm granulations (more than 20% of cells are damaged and the genetic material is fragmented), this indicates a toxic effect of the investigated compounds. The microscopic evaluation results are then subjected to statistical calculations. The final result is presented in a graphic form, showing the two variants of the test (with and without S9 mix) for comparison. If the results are higher than those obtained from the positive control, this indicates genotoxicity of the studied chemical.

INTERPRETATION OF *IN VITRO* MN TEST RESULTS

An *in vitro* micronucleus test with cytochalasin-B permits the observation of different types of cells. It is possible to count not only micronuclei which are involved in chromosome breakage and chromosome loss response, but also other chromosome rearrangements. The appearance of nucleoplasmatic bridges (NPBs), which are the result of dicentric chromosomes or nucleoplasmid buds (NBUDs) – a gene amplification effect as well as necrosis and apoptosis, can be used as biomarkers within the CBMN (Fenech 2007).

During interpretation of the results in the *in vitro* micronucleus test with V79 cells, it is necessary to choose the appropriate cells which may be scored. Due to the fact that the micronucleus test developed for human lymphocytes was adapted to different cells, V79 cells were characterized according to the guidelines in human lymphocytes proposed by Fenech (2000).

In the cytokinesis block micronucleus test the observable cells that may be scored should be binucleated (BN) with two distinctly separate nuclei. The two main nuclei should have intact nuclear membranes and should be situated within the same cytoplasmic boundary. They may touch each other, although ideally they should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable. The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells. What plays an

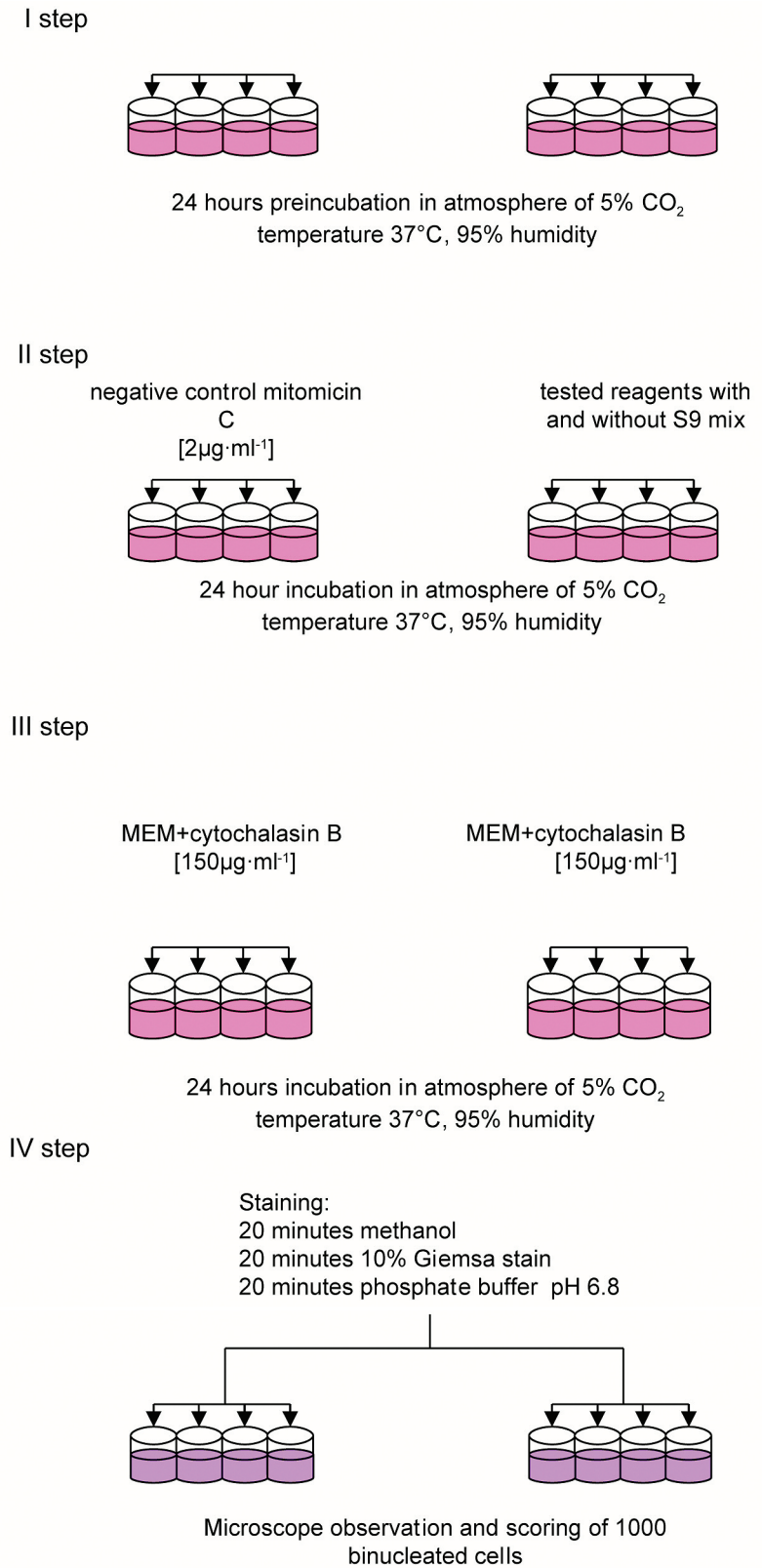

Figure 4. Scheme of *in vitro* micronucleus test.

important role here is the size, staining and location of nuclei. They should be approximately equal in size, staining pattern and strength. An example of the type of binucleated V79 cells that may be scored is illustrated in Figure 5.

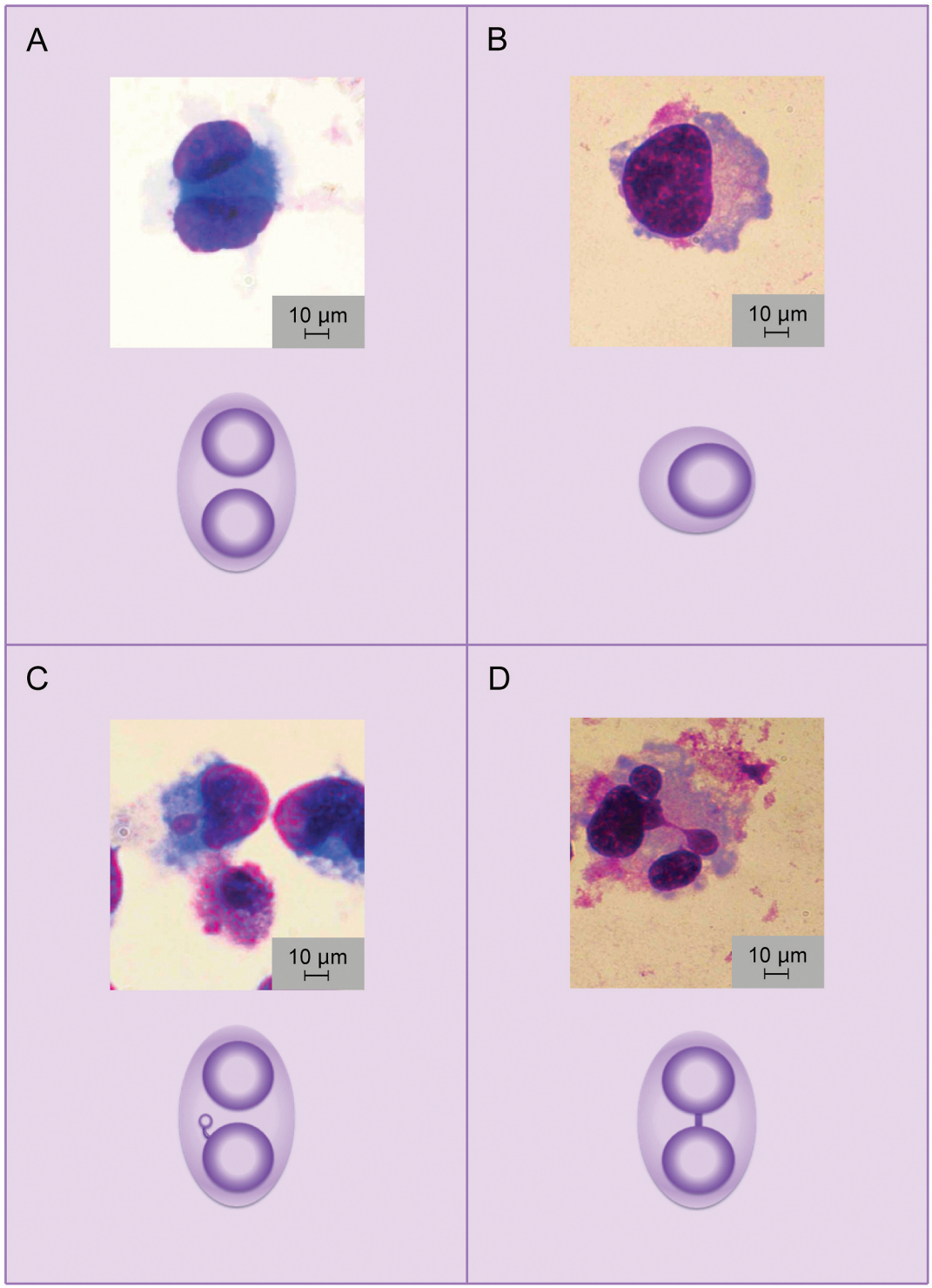

Figure 5. The types of nuclear formations in V79 cells observed using the *in vitro* MN test. A) Correctly binucleated cell with distinctly two separate nuclei. B) Normally mononucleated cell. C) Binucleated cell with nuclear blebs. D) Binucleated cell with a narrow nucleoplasmic connection between the main nuclei, which must not be counted for MN frequency.

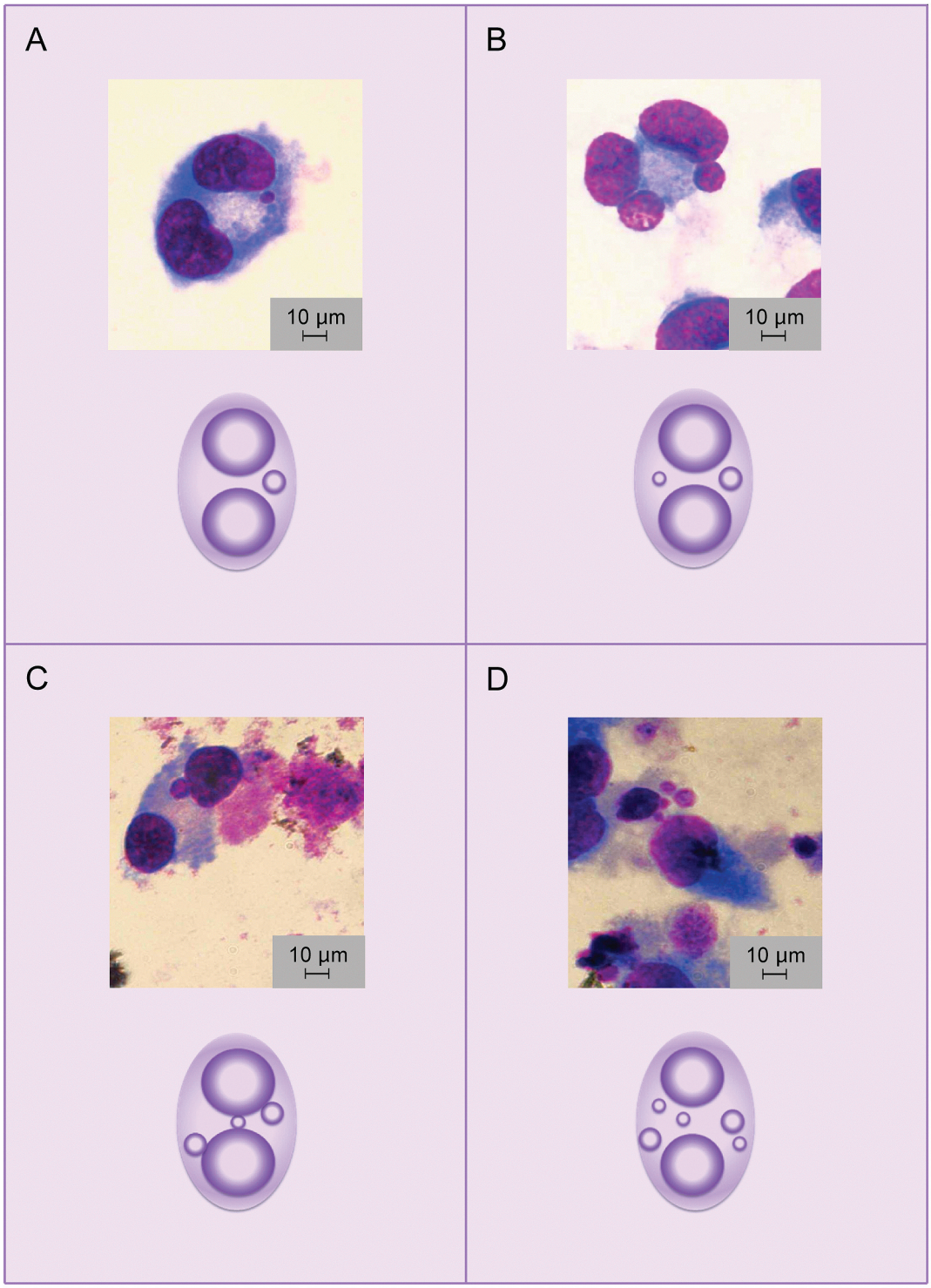

Figure 6. The types of binucleated V79 cells with micronuclei in the *in vitro* MN test. A) Correctly binucleated cell with one micronucleus as a result of aneugenic or clastogenic response. B) Correctly binucleated cell with two micronuclei. Binucleated cells with micronucleus overlap the main nuclei. C) and D) Binucleated cells with more than two micronuclei. Such cells must not be counted for MN frequency.

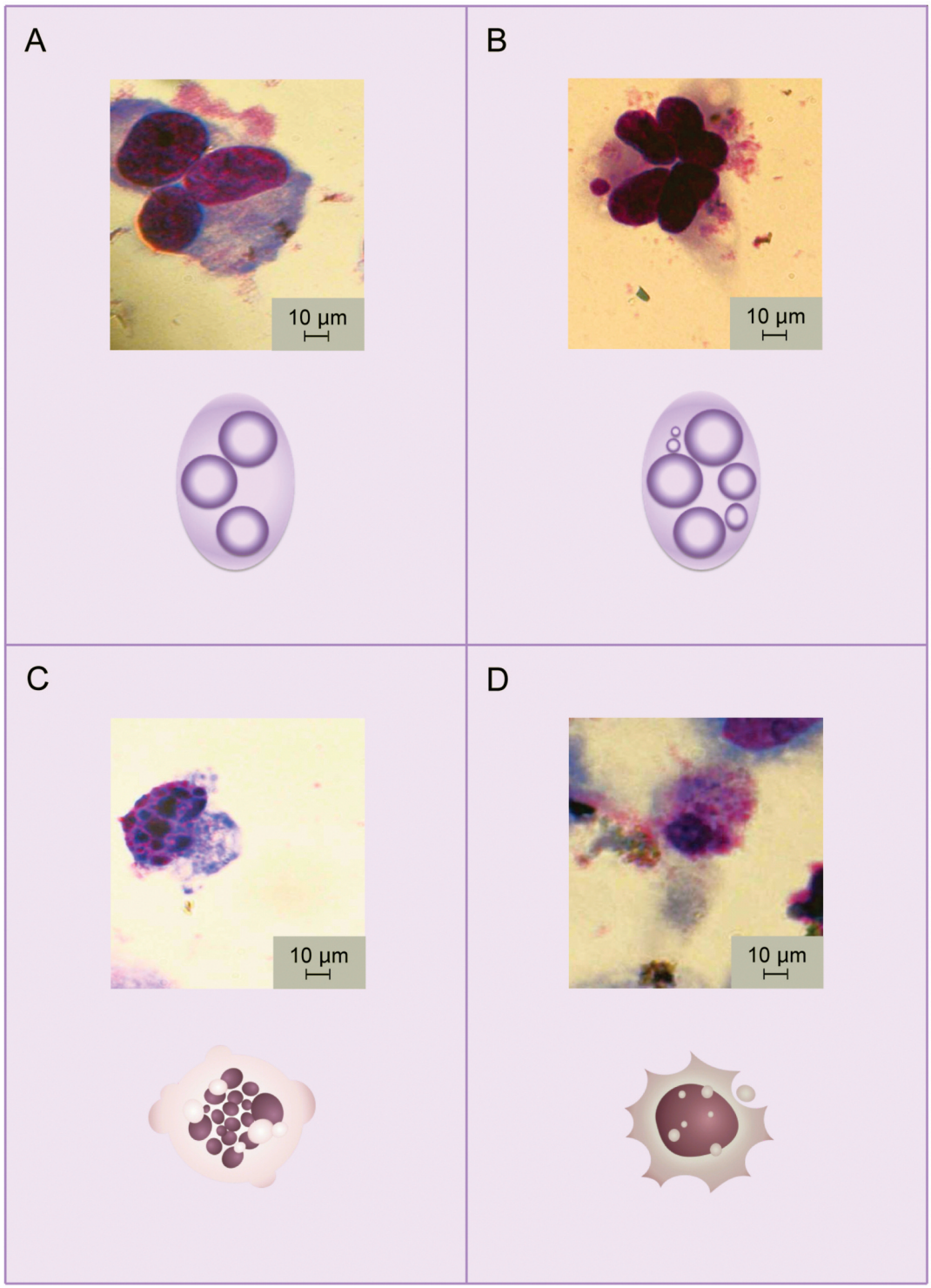

Figure 7. Other types of V79 cells observed in the *in vitro* MN test. A) Cell with tree nuclei. B) Multinucleated cell. C) Apoptotic cell, showing chromatin condensation, intact cytoplasm and nuclear boundaries; also cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane. D) Necrotic cell, exhibiting a pale cytoplasm with numerous vacuoles and damaged cytoplasmic membrane with a fairly intact nucleus; also cells exhibiting loss of cytoplasm and damaged/irregular nuclear membrane with a partially intact nuclear structure.

The cells which were exposed to polycyclic aromatic compounds responded in the form of formation of morphologically identical, but smaller, nuclei - micronuclei (MNi). Many variants of such cells can be observed but not all of them result from the test. Some of these cells have formations which only resemble micronuclei. Examples of typical MNi which occur in the V79 cells are shown in Figure 6.

The correct MNi (Figure 6) may touch, but not overlap, the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary. They should not be linked or connected to the main nuclei. The normal MNi usually have the same staining intensity as the main nuclei, although staining may occasionally be more intense.

Frequently, among the cells exposed to polycyclic aromatic compounds, other types of cells can also observed which should not be scored for MN frequency. These cell types include various multi-nucleated cells or cells which are necrotic or apoptotic (Figure 7).

Occasionally, the two nuclei within a BN cell are attached by a fine nucleoplasmic bridge (Figure 5D) or binucleated cells containing structures which resemble micronuclei, although they should not be scored as micronuclei originating from chromosome loss or chromosome breakage. It is possible to observe a trinucleated cell in which one of the nuclei is relatively small (Figure 7A) but has a diameter greater than 1/3 of the diameter of the other nuclei or dense stippling in a specific region of the cytoplasm.

What should be ignored is any extruded nuclear material which appears like a micronucleus with a narrow nucleoplasmic connection to the main nucleus and nuclear blebs that have an obvious nucleoplasmic connection with the main nucleus (Figure 5). These cells should not be scored for micronucleus frequency.

CONCLUSIONS

In recent years, the *in vitro* micronucleus test has become an attractive tool for genotoxicity testing (chromosome damage endpoints) because of its capacity to detect both clastogenic and aneugenic events, simplicity of scoring, accuracy, multi-potentiality, wide applicability in different cell types. The toxicological relevance of the MN test is strong for several key reasons:

- it covers several endpoints,
- has high sensitivity,
- its predictivity for *in vivo* genotoxicity requires adequate selection of cell lines,
- its statistical power is increased by the high throughput methodologies made available recently,
- it may become a possible candidate for replacing *in vivo* testing,
- it allows good extrapolation for potential limits of exposure or thresholds (Kirsch-Volders et al. 2011).

This method has also proven to be suitable for investigating the polycyclic aromatic hydrocarbons. The use of an appropriate cell line and a variant of the cytokinesis block in the *in vitro* micronucleus assay allows the examination of the properties of a variety of PAHs.

However, the *in vitro* MN test does not provide information about the origins of micronuclei (Corvi et al. 2008). Only in combination with the available methods, such as fluorescence in situ hybridizations (FISH), does it provide the possibility of deeper analysis of the mechanism of formation of micronuclei. Therefore, further research should be focused on precisely distinguishing clastogen from aneugen activity with the available methods. When used in combination with FISH using probes labelling the pan(peri)centromeric region of chromosomes, it can distinguish between micronuclei containing a whole chromosome and an acentric chromosome fragment.

Moreover, the *in vitro* micronucleus test in conjunction with another *in vitro* assay, e.g. a bacterial gene-mutation test (such as an Ames test), can cover all of the essential mutagenic endpoints: gene mutations, structural chromosome damage and aneuploidy (Kirkland et al. 2011). Thus, the results of an *in vitro* micronucleus test can complement to the existing knowledge of the properties of PAHs.

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