Cytokinesis-block micronucleus (CBMN) assay/CBMN cytome assay in human lymphocytes after *in vitro* irradiation and its use in biodosimetry

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Introduction

Cytogenetic methods have become a valid tool to assess radiation damage and to support triage, medical treatment decisions and prognosis of radiation casualties. These methods are relatively cheap and easy to perform but indispensable laboratory back-up along with time delay to acquire first results of cytogenetic assessment make these methods unsuitable for military field conditions. Recently, several new biodosimetric methods overcoming some of these limitations have been proposed, including protein marker (e.g. H2AX) or serum protein analyses (1, 2, 3). Nevertheless, these assays are in development and have not yet been tested in a real triage situation (3). Therefore, cytogenetic methods still remain a gold standart of biodosimetry.

The *in vitro* cytokinesis-block micronucleus (CBMN) assay is a cytogenetic method based on the assessment of micronuclei in nucleated cells that have completed only one nuclear division (4, 5).

Over the past few years, the CBMN test has evolved into a more comprehensive method for measuring DNA damage, cytostasis and cytotoxicity and is collectively defined as the cytokinesis-block micronucleus cytome assay (CBMN cytome assay; 6, 7, 8). Apart from the evaluation of micronuclei, the CBMN cytome assay allows to assess other relevant biodosimetric markers: nucleoplasmic bridges (NPBs), nuclear buds (NBUDs), proportion of dividing cells (parameter of cytostasis), and cells undergoing apoptosis and necrosis (parameters of cytotoxicity) (6, 7).

Herein, we present the results of six endpoints - Micronuclei (MN), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs) measured in binucleated (BN) cells, MN in measured mononucleated cells, nuclear division index (NDI), and amount of death cells (apoptotic and necrotic cells) assessed by the CBMN cytome assay in human blood lymphocytes after *in vitro* irradiation.

Material and Methods

Sampling

Human blood was collected from 6 healthy donors [4 males and 2 females aged 26 – 29 (mean 27.2 ± 0.9), non-smokers]. No medication was taken by the donors for at least 1 month before sampling. Blood (10 ml) from each donor was collected into a plastic tube with 0.2 ml Li-Heparin (Zentiva, Prague, Czech Republic).

Irradiation

Only 6 ml of heparinized blood from each donor divided into 6 1ml parts was used. One part as a control and the others for exposure to 1, 2, 3, 4, and 5 Gy. The samples were irradiated using 60Co unit (Chirana, Prague, Czech Republic) at a dose rate of 0.83 Gy/min with a target distance of 1 m.

Cell culture, fixation, slide preparation and staining

Cells cultures, fixation and slide preparation was performed according the Internation Atomic Energy Agency (IAEA) manual: Cytogenetic Analysis for Radiation Dose Assessment (11).

In short, whole blood cultures were prepared by adding 1 ml blood to 9 ml culture RPMI-1640 culture medium (Sigma-Aldrich, Prague, Czech Republic) supplemented with 20% fetal bovine serum (PAA Laboratories GmbH, Austria), 2 mmol/l glutamine (Sigma-Aldrich), 100 UI/ml penicillin (Sigma-Aldrich) and 0.1 mg/ml streptomycin (Sigma-Aldrich). Phytohemagglutinin (PHA) at a concentration of 10 ul/ml was used to stimulate lymphocyte proliferation. The blood was cultured in tissue culture vessels at 37°C, 5% CO2 in a humidified atmosphere. Cytochalasin B (Sigma-Aldrich Company, Prague, Czech Republic) was added 44 h after PHA stimulation at a concentration of 6 ug/ml to block cells at cytokinesis.

After a 72h incubation period, cells were collected by centrifugation (800 rpm, for 5 min), hypotonically treaded with cold (4 ºC) 0.075M KCl to lyse red blood cells, and fixed with a fixative solution containing methanol:acetic acid (3:1) with 1% formaldehyde. The cells were washed with two further exchanges of fixative solution, this time without formaldehyde. After fixation, the cells were gently resuspended, dropped onto wet clean glass slides and allowed to dry.

Slides were stained in 4% Giemsa solution for 8 min (Sigma-Aldrich).

Slide evaluation

Stained samples were evaluated using a BX-51 microscope (Olympus, Prague, Czech Republic) at 400fold original magnification. A total of 1000 binucleated cells was evaluated for the frequency of MN, NPBs and NBUDs. A total of 500 mononucleated was examined for the frequency of MN. A total of 500 living interphasic cells was used for assessment of mono-, bi-, and poly-nucleated cells and calculation of NDI. Finally, 500 cells were used to assess the amount of death cells (necrotic and apoptotic cells).

Scoring criteria

For scoring, modified criteria presented by Fenech et al. (2003) (10) and IAEA manual (11) were used. These criteria were standartised for the CBMN cytome assay using lymphocyte separation procedure prior the step of cell cultivation and therefore missing the hypotonic treatment. Hypotonic treatment often destroys the outer membrane and only nuclei remain. Thus, nuclei in close proximity with similar staining intensity, staining patern (distribution of eu- and hetero-chromatin), and approximately of the same size were considered as originating from 1 cell.

Criteria for selection of binucleated cells which can be scored for the presence of MN, NPBs and NBUDs (see Figure 1 - 5):

The cells should be binucleated (BN).

The two nuclei in a BN cell should have intact nuclear membranes.

The two nuclei in a BN cell should be approximately equal in size, staining pattern and staining intensity.

The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge wich is no wider than one-fourth of the largest nuclear diameter.

The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.

If a cell/nuclei are localized within a cluster of cells in which the situation is not synoptical, it is excluded from the count.

Criteria for scoring MN (see Figure 2, 3, 4, and 5):

The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively.

MN are round or oval in shape.

MN are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles.

MN are not linked or connected to the main nuclei.

MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

MN usually have the similar staining intensity as the main nuclei but occasionally staining may be less or more intense.

Criteria for scoring NPBs (see Figure 4 and 5):

NPBs are a continuous nucleoplasmic link between the nuclei in a binucleated cell.

The width of a nucleoplasmic bridge may vary considerably but usually does not exceed one-fourth of the diameter of the nuclei within the cell.

NPBs should have the same staining characteristics of the main nuclei.

On rare occasions more than one NPB may be observed within one binucleated cell.

A binucleated cell with a nucleoplasmic bridge may or may not contain one or more micronuclei.

Criteria for scoring NBUDs (see Figure 3):

NBUDs are a distinct protuberance of nuclear membranes.

NBUDs may resemble a MN attached to the main nuclei with a nucleoplasmatic bridge.

NBUDs should have the same staining characteristics of the main nuclei.

On rare occasions more than one NBUD may be observed within one binucleated cell.

A binucleated cell with a NBUD may or may not contain one or more micronuclei.

For cell cycle analysis,

500 cells per treatment group were scored for the presence of one, two or more than two nuclei (see Figure 6) and the nuclear division index (NDI) was calculated as follows: NDI = [1N + (2 × 2N) + (4 × >2N)]/C, where 1N is number of cells with one nucleus, 2N — with two nuclei, and >2N — with more than two nuclei, C — number of cells examined (12).

Criteria for scoring death cells (see Figure 3 and 7):

Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact nuclear boundaries.

Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies [rarely within an intact cytoplasm/cytoplasmic membrane (if present)]. Staining intensity in the nucleus and nuclear fragments is usually greater than in viable cells.

Late necrotic cells exhibit damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary. Staining intensity of the nucleus and cytoplasm is usually less than that observed in viable cells.

Early necrotic cells can not be identified due to the use of hypotonic treatment since the presence of cytoplasm is necessary for their identification. Early necrosis can be identified by the presence of a pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and damaged cytoplasmic membrane with a fairly intact nucleus.

The Mann-Whitney test was used for the statistical analysis giving mean ± 2 × standard error of mean (S.E.M.). The differences were considered significant when $p \leq 0.05$.

Statistical analysis

Results

The results of the frequencies of MN, NPBs and NBUDs measured in BN cells after irradiation by 1, 2, 3, 4, and 5 Gy are summarized in Table 1. In comparison with control values, MN increased 4.8-, 11.0-, 21.3-, 36.7-, and 52.7-fold, NPBs produced 3.5-, 6.5-, 12.0-, 17.0-, and 20.0-fold increase and NBUDs were 3.2-, 5.0-, 7.2-, 10.2-, and 13.0-fold higher, respectively.

Table 1. Average values of MN, NPBs, NBUDs measured in 1000 BN cells ± 2 × S.E.M.

Table 1. Average values of MN, NPBs, NBUDs measured in 1000 BN cells ± 2 × S.E.M.

Dose (Gy)	0	1	2	3	4	5
MN	20 ± 1	95 ± 8 [2]	219 ± 11 [2b]	426 ± 22 [2b]	734 ± 29 [2b]	1053 ± 50 [2b]
NPBs	2 ± 1	7 ± 3 [2]	13 ±6 [2]	24 ± 12 [2]	34 ± 14 [2]	40 ± 15 [2]
NBUDs	5 ± 2	16 ± 5 [2]	[2]25 ± 4 [2a]	36 ± 6 [2]	51 ± 11 [2]	65 ± 11 [2]

Significant differences among non-irradiated and irradiated animals: p≤0,05 - 1, p≤0,01 - 2, p≤0,001 - 3.
Significant differences among values of animals irradiated by the closest lower dose: p≤0,05 - a; p≤0,01 – b; p≤0,001 - c.
Significantly higher frequency of MN measured in 500 mononucleated cells was observed after irradiation by 1, 2, 3, 4, and 5 Gy. The values showed 3.0-, 6.5-, 9.0-, 14.0-, and 20.5-fold increase when compared with control value (Table 2)

Table 3. Amount of mononucleated, binucleated, and polynucleated cells measured in 500 living interphasic cells ± 2 × S.E.M.

Dose (Gy)	0	1	2	3	4	5
MonoN	287 ± 27	318 ± 18	333 ± 23 [1]	357 ± 19 [2]	368 ± 22 [2]	384 ± 11 [2]
BN	172 ± 19	148 ± 19	138 ± 21 [1]	120 ± 19 [1]	113 ± 22 [2]	103 ± 11 [2]
PN	43 ± 8	34 ± 3	29 ± 4 [1]	24 ± 3 [2]	19 ± 2 [2a]	13 ± 3 [2a]

Significant differences among non-irradiated and irradiated animals: p≤0,05 - 1, p≤0,01 - 2, p≤0,001 - 3.
Significant differences among values of animals irradiated by the closest lower dose: p≤0,05 - a; p≤0,01 – b; p≤0,001 - c.

Table 2. Average values of MN measured in 500 mononucleated cells ± 2 × S.E.M.

Dose (Gy)	0	1	2	3	4	5
MN	2 ± 1	6 ± 3 [1]	13 ± 3 [2a]	18 ± 5 [2]	28 ± 5 [2a]	41 ± 5 [2b]

Significant differences among non-irradiated and irradiated animals: p≤0,05 - 1, p≤0,01 - 2, p≤0,001 - 3.
Significant differences among values of animals irradiated by the closest lower dose: p≤0,05 - a; p≤0,01 – b; p≤0,001 - c.

Table 4. NDI ± 2 × S.E.M.

Dose (Gy)	0	1	2	3	4	5
NDI	1,60 ± 0,08	1,50 ± 0,04 [1]	1,45 ± 0,06 [2]	1,38 ± 0,05 [2]	1,34 ± 0,05 [2]	1,28 ± 0,02 [2]

Significant differences among non-irradiated and irradiated animals: p≤0,05 - 1, p≤0,01 - 2, p≤0,001 - 3.
Significant differences among values of animals irradiated by the closest lower dose: p≤0,05 - a; p≤0,01 – b; p≤0,001 - c.

Table 5. Amount of death cells measured in 500 cells ± 2 × S.E.M.

Dose (Gy)	0	1	2	3	4	5
Death cells	105 ± 13	122 ± 15	131 ± 22	129 ± 12	141 ± 24 [1]	153 ± 19 [2]

Significant differences among non-irradiated and irradiated animals: p≤0,05 - 1, p≤0,01 - 2, p≤0,001 - 3.
Significant differences among values of animals irradiated by the closest lower dose: p≤0,05 - a; p≤0,01 – b; p≤0,001 - c.

Discussion

In this study, we present the results of six endpoints of CBMN cytome assay measured in human blood lymphocytes after *in vitro* irradiation. Parameters of genotoxicity (MN, NPBs and NBUDs in binucleated cells and MN in mononucleated cells) and cytostasis (NDI) were significantly and dose-dependently changed after irradiation by 1 – 5 Gy. On the other hand, the parameter of cytotoxicity (amount of dead cells) was significantly increased only after irradiation by the dose of 4 and 5 Gy. The results indicate that all parameters evaluated by CBMN cytome assay could be used in biodosimetry but only parameters which show statistical differences and no overlapping of the 95% confidence intervals between neighbouring doses could be practically used to distinguish each particular dose. According to our results, only one parameter matches these criteria – MN measured in binucleated cells. Therefore, the parameter was used to construct *in vitro* linear-quadratic dose-response calibration curve (see Graph 1) since it is customary for each laboratory carring out cytogenetic biodosimetry to establish and use its own calibration curve (3) due to high interlaboratory differences (10, 11, 13). This calibration curve could be used as a biodosimetric tool for triage of people accidentally exposed to ionizing radiation.

From practical point of view, the CBMN assay (assessment of MN in BN cells) is an easy and cost-effective method suitable and sufficient enough to distinguish those who are severely exposed (> 1 Gy) and require early medical treatment from those less exposed during radiation accidents including situations of acute high dose whole body external exposure, protracted and fractionated external exposure (14) as well as protracted exposure after incorporation of long lived radionuclides (15).

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The method has three major limitations. First, its sensitivity is limited to 0.2 Gy which makes the method insufficient for the purposes of radiation hygiene. The occupational irradiation rarely exceeds the annual dose limit of 50 mSi/year (annual occupational dose limit of total effective dose equivalent for adults). This disadvantage is related to relatively high and variable spontaneous MN yield (5). According to our results, the MN background level is 20 (20.17) which is in agreement with IAEA manual reporting the background MN values to range from 2 to 36 per 1000 BN cells (13) and other studies (5, 8, 12, 16). The sensitivity of CBMN assay can be substantiallly increased in the low-dose range if pancentromeric p82H probe is applied to distinguish between MN containing acentric fragments and those containing whole chromosomes (5, 18, 18, 19). Even though this variant is more time-demanding and laborious due to the step of probe hibridisation, nevertheless, Pala et al. (19) showed a 0.05Gy detection limit when 2000 BN cells were evaluated. This variant of CBMN assay may play an important role in the field of radiation protection in the future. Second, the method can not be used to determine the fraction of the body exposed to ionizing radiation since the data show overdispersion even in the case of a total body irradiation (5). Finally, the necessity of laboratory equipment and time delay between receiving the blood sample by laboratory and first results make the method less suitable for field conditions. This limitation is common for all cytogenetic methods - dicentric analysis, translocation analysis, or premature chromosome condensation (PCC) method (20), which are used in biodosimetry. Running a CBMN assay requires a 72 hour air condition-controlled cultivation, several hours for fixation, slide preparation, and staining and according to our experience, 1 hour on average is needed for evaluation of 1 slide.

The evaluation procedure can be accelerated if an automated image analysis system is used. Recently, effective automatic evaluation hardware and software (Metafer 4) has been developed by Metasystems. The system enables evaluation of 120 slides in a 12 hour shift (5).

Other parameters measured by CBMN cytome assay show biodosimetric potential but their use is limited. Particularly NPBs have been suggested as very perspective marker due to low control values (1.83 according to our results) and their relation to dicentric chromosomes and dicentric analysis, respecitvely (21). Dicentric analysis, which evaluates frequency of dicentric chromosomes in mitotic spreads, enables to detect genotoxic damage produced by doses as low as 0.05 Gy when 1000 mitotic spreads are assessed. This requires 2 man working days for a skilled evaluator (22). The evaluation of NPBs makes the assessment of dicentric chromosomes easier and time-comparable to CBMN assay. Nevertheless, we did not observed statistical differences between 1- and 2-, 2- and 3-, 3- and 4-, and 4- and 5Gy group and the 95% confidence intervals between neighbouring doses overlap, which is similar for evaluation of NBUDs in BN cells, MN in mononucleated cells as well as NDI. This indicates that the assessment of NPBs and the remaining parameters is only additional to CBMN assay and should be avoided in large scale accidents, in which fast evaluation of many samples is necessary since CBMN assay requires 1 hour per slide but the whole CBMN cytome assay needs approximately 3 hours to evaluate 1 slide.

Conclusion

Our results suggest that micronuclei measured in binucleated cells are the best biomarker of ionising radiation evaluated by CBMN cytome assay and assessment of other parameters is possible but their use is limited due to insignificant differences and overlapping of the 95% confidence intervals between neighbouring doses. Values of micronuclei measured in binucleated cells were used to construct *in vitro* linear-quadratic dose-response calibration curve which means that being equiped with a flow cytometer, our laboratory is currently capable of comprehensive dose assessment of victims of radiation accidents and can effectively offer this service to Integrated Rescue System.

Figures:

Figure 1

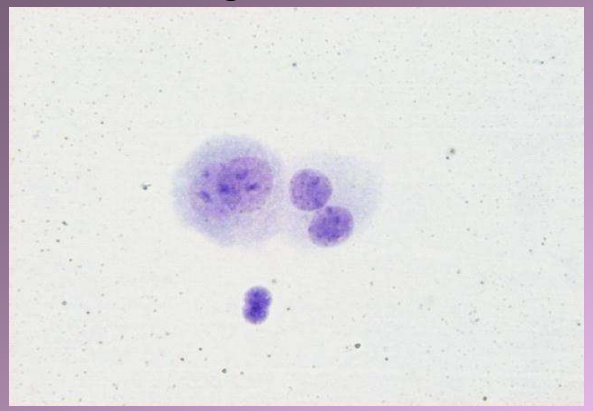

Figure 2

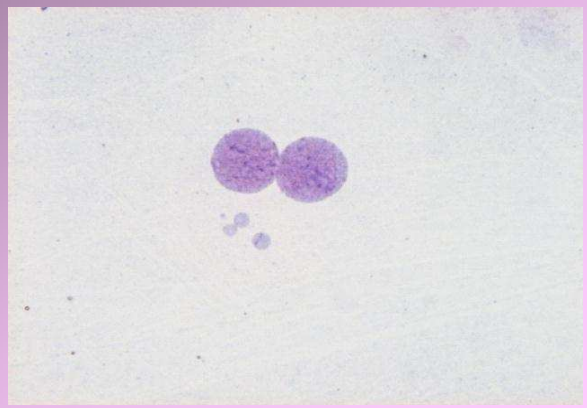

Figure 3

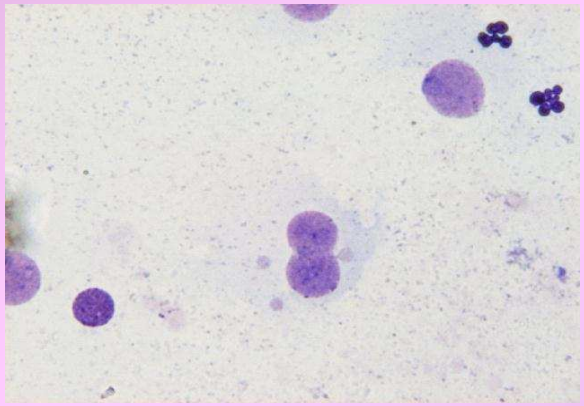

Figure 4

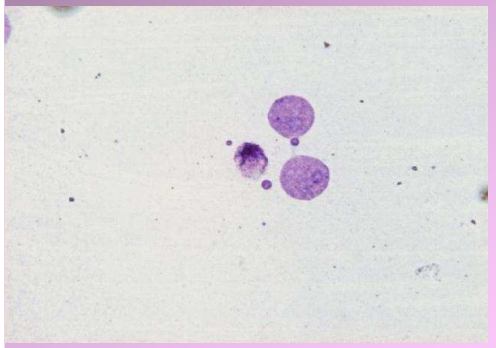

Figure 5

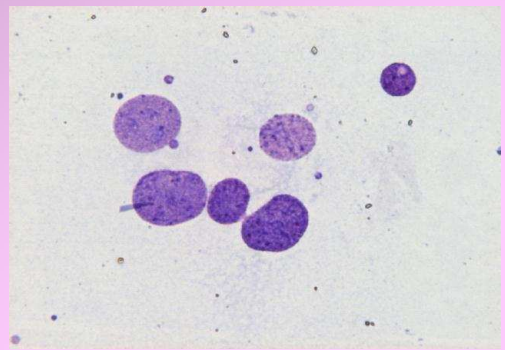

Figure 6

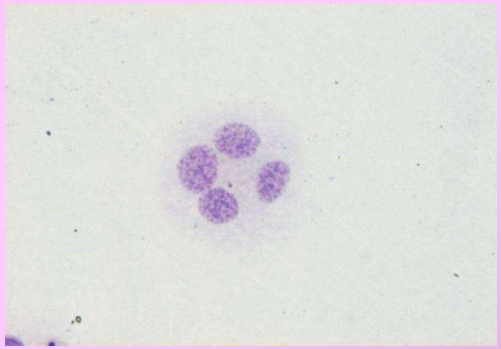

Figure 7

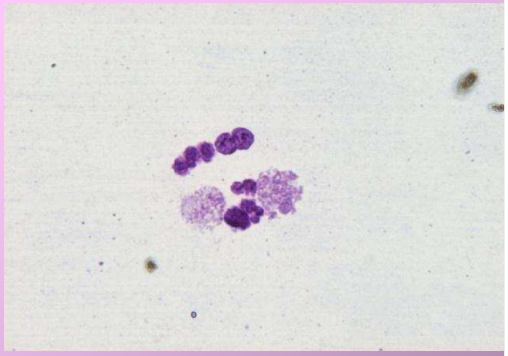

Figure 1. Sample of sham-irradiated human blood at 400-fold magnification.
(1) binucleated cell with clearly separated nuclei, cytoplasm is present
(2) binucleated cell with cytoplasm, nuclei are overlayed but both have intact nuclear membranes.
(3) „cell“ without cytoplasm. The nuclear remains represent either a one deforemed nuclei or two nuclei with unseparated nuclear membranes. For the purpose of this study such a cell was counted as mononucleated.
Figure 2. Sample of human blood after irradiation by 5 Gy at 400-fold magnification. The picture shows a binucleated cell with intact nuclear membranes and 4 micronuclei.
Figure 3. Sample of human blood after irradiation by 5 Gy at 400-fold magnification.
(1) binucleated cell without cytoplasm. Solid arrows point out micronuclei and dashed arrow marks a nuclear bud.
(2) mononucleated cells without cytoplasm and without micronuclei.
(3) late apoptotic cells exhibiting nuclear fragmentation into smaller nuclear bodies.
Figure 4. Sample of human blood after irradiation by 5 Gy at 400-fold magnification. The figure shows a binucleated cell with a micronucleus laying over a nucleoplasmic bridge (dashed arrow) and another two micronuclei (solid arrows).
Figure 5. Sample of human blood after irradiation by 5 Gy at 400-fold magnification.
(1) binucleated cell with a nucleoplasmic bridge (solid arrow) and a micronucleus (dashed arrow).
(2) mononucleated cells with micronuclei.
(3) mononucleated cells without micronuclei.
Figure 6. Sample of human blood after irradiation by 5 Gy at 400-fold magnification showing a tetranucleated cell with cytoplasm and micronucleus. Micronuclei, nucleoplasmic bridges and nuclear buds present in tri- and tetranucleated cells were not evaluated.
Figure 7. Sample of human blood after irradiation by 5 Gy at 400-fold magnification.
(1) early apoptotic cell with increased chromatin condensation within the nuclei with intact nuclear boundaries.
(2) late apoptotic cells exhibiting nuclear fragmentation.
(3) necrotic cells with damaged/irregular nuclear membrane. Staining intensity of the nucleus and cytoplasm is less than that observed in viable cells.

- **Reference**

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