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Selection and optimization of staining techniques of Chinese hamster lung fibroblast V79 cell preparations

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ABSTRACT

We present the results of optimization of fixation and staining of the Chinese hamster lung fibroblast V79 cell line. The experiment included four methods of fixation and three methods of staining and enabled the selection of the best visualization technique for V79 cell lines culture. The Schiff method of colouring with Light Green contrasted well the cell nucleus and the cytoplasm, but the staining was light-sensitive and transient. The Giemsa staining made cell preparations suitable for microscopic evaluation, although the difference between the nucleus and the cytoplasm was not always easily recognisable.

INTRODUCTION

Microscope images of cells are often unclear and lacking contrast, which is associated with the blandness of cells. To obtain better contrast and visibility of the entire cell and its organelles, various types of staining must be applied. There are many different staining techniques preceded by fixation. The choice of the method depends on the type of test and expected results (http://brain.fuw.edu.pl/edu/ BIOL:Wst% C4% 99p._Mikroskopia_oraz_technika_histologiczna).

We study genotoxicity of a variety of chemical compounds using the technique of the *in vitro* micronucleus test on cell line V79. This technique needs scoring of the percentage of cells with micronuclei, which is based on identification of many thousands of cells in each test. In an effort to make the cell scoring easier and to simplify the assessment of the results, we had designed this experiment in order to select the best staining techniques based on such criteria as: the contrast between cytoplasm and nuclei and the photostability of a dye.

All methods were based on the ISO 21427-2, Water Quality – Evaluation of Genotoxicity by Measurement of Induction of Micronuclei - Part 2: Mixed Population Method using the V79 Cell Line.

MATERIAL

1. Lung fibroblasts cell line V79 Chinese hamster (*Cricetulus griseus*), DSMZ - Deutsche Sammlung von Mikroorganismen Zellkulturen GmbH und / Human and Animal Cell Lines Database,
2. MEM growth medium supplemented with L-glutamine (Earlea salts, amino acids, phenol red) Minimum Essential Medium SIGMA No. 51411C,
3. Bovine blood serum FBS (Fetal Bovine Serum), Sigma No. F6178,
4. Trypsin (0.25% solution of EDTA), Sigma No. T4049,
5. Antibiotics (Streptomycin, Penicillin Sigma No. P4333, Amphotericin B Sigma No. A2942),
6. Giemsa dye solution (0.5g of powdered sulphate fuchsin, 25ml glycerin, 75ml of methanol) Sigma No. G9641,
7. Phosphate buffer pH 6.8 (1000ml distilled water, 0.71g Na_2HPO_4, 0.68g KH_2PO_4),
8. Schiff's reagent ($C_{14}H_{14}ClN_9S$) SIGMA No. 3952016,
9. Cytoplasmic dye Light Green ($C_{37}H_{34}N_2Na_2O_9S_3$) Sigma No. L5382,
10. DPX SIGMA No. 44581,
11. Glacial acetic acid (~98%) POCH (Polish Chemical Reagents) No. 568765428,
12. Ethyl alcohol (~96%) POCH No. 396420420,
13. Methanol (~98%) POCH No. 621990426,
14. Acetone (~98%) POCH No. 102480411.

METHODS

Cell culture

Cells were grown in polyethylene bottles with a breeding area of 25cm^2 in a medium consisting of MEM with the addition of L-glutamine containing 10% FBS and 1% antibiotics. The cells were cultured for 48 hours at 37°C, 5% CO_2 and humidity at 95%. After incubation, the cells were transferred onto a four-hole plate and cultured under the same conditions as above, i.e. $2.5 \cdot 10^4$ cells in 1ml per well. The cells were subjected to 48-hour incubation (based on ISO 21427-2).

Fixation with a mixture of ethyl alcohol and acetic acid

A fresh mixture was prepared in a ratio of 3:1, i.e. three volumes of ethanol and one volume of glacial acetic acid. Fixation involved immersing the preparations in a Coplin container for 10 minutes. The preparations were then allowed to dry at room temperature in a horizontal position without cover. The preparations were left for 24 hours under a laminar chamber (without forced air flow).

Fixation with methanol

The preparations were fixed with methanol at room temperature for 20min in Coplin containers. They were then allowed to dry at room temperature in a horizontal position without cover. The preparations were left for 24 hours under a laminar chamber (without a forced air flow).

Fixation with methanol and acetone

The preparations were left in Coplin containers at reduced temperature (4°C), first for 3 minutes in chilled methanol (4°C) and then 3 minutes in chilled acetone (4°C). They were then allowed to dry uncovered at room temperature in a horizontal position and were left for 24 hours under a laminar chamber (without a forced air flow).

Fixation with ethanol

Fixation involved immersing the culture plates in a Coplin container, in 96% ethanol for 20 minutes. They were then allowed to dry uncovered at room temperature in a horizontal position and were left for 24 hours under a laminar chamber (without a forced air flow).

Giemsa staining

- A phosphate buffer was prepared with 1000ml of distilled water, 0.71g Na_2HPO_4 and 0.68g K_2HPO_4. The solution was then subjected to autoclaving for 15 minutes at 121°C,
- A preparation of a 10% solution of Giemsa stain: 0.5g of powdered Giemsa dye was added to a solution of 25ml of anhydrous glycerine and 75ml of methanol,
- Staining with a 10% solution for 20 minutes,
- After staining, the preparations were rinsed with a phosphate buffer at pH 6.8 for 20 minutes to remove excess Giemsa dye,
- The preparations were left to dry in a horizontal position, covered, at room temperature, for 24 hours.

Schiff staining with Light Green

- Washing the preparations with 50% ethanol for 1 minute,
- Washing with 20% ethanol for 1 minute,
- Washing with distilled water for 2 minutes,
- Rinsing with 5M HCl for 30 minutes,
- Washing with tap water,
- Drying preparations, without total drying,
- Rinsing preparations in Schiff's reagent for 60 minutes in the dark,
- Rinsing with tap water followed by distilled water for 5 minutes,
- Washing the preparations in a Light Green dye for 20-30 seconds,
- Rinsing with distilled water,
- Leaving the preparations to dry uncovered in a horizontal position, at room temperature for 24 hours.

Schiff staining

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- Washing with 20% ethanol for 1 minute,
- Washing with distilled water for 2 minutes,
- Rinsing with 5M HCl for 30 minutes,
- Washing with tap water,
- Drying the preparations, without total drying,
- Soaking the preparations in Schiff's reagent for 60 minutes in the dark,
- Rinsing with distilled water,
- Leaving the preparations to dry in a horizontal position, covered, at room temperature for 24 hours.

Closing of slides

All preparations after fixation and staining were closed histologically with DPX. Drops of reagent were applied to each well, then covered with a glass cover so that no air bubbles were formed. The preparations were then allowed to dry in a horizontal position, under cover, for 48 hours at room temperature.

RESULTS

The fixation methods used in this experiment (a solution of ethyl alcohol and acetic acid; methyl alcohol, a solution of methyl alcohol and chilled acetone; 96% ethyl alcohol) showed no differences in the degree of cell dehydration and did not damage or disturb cell adhesion to the substrate.

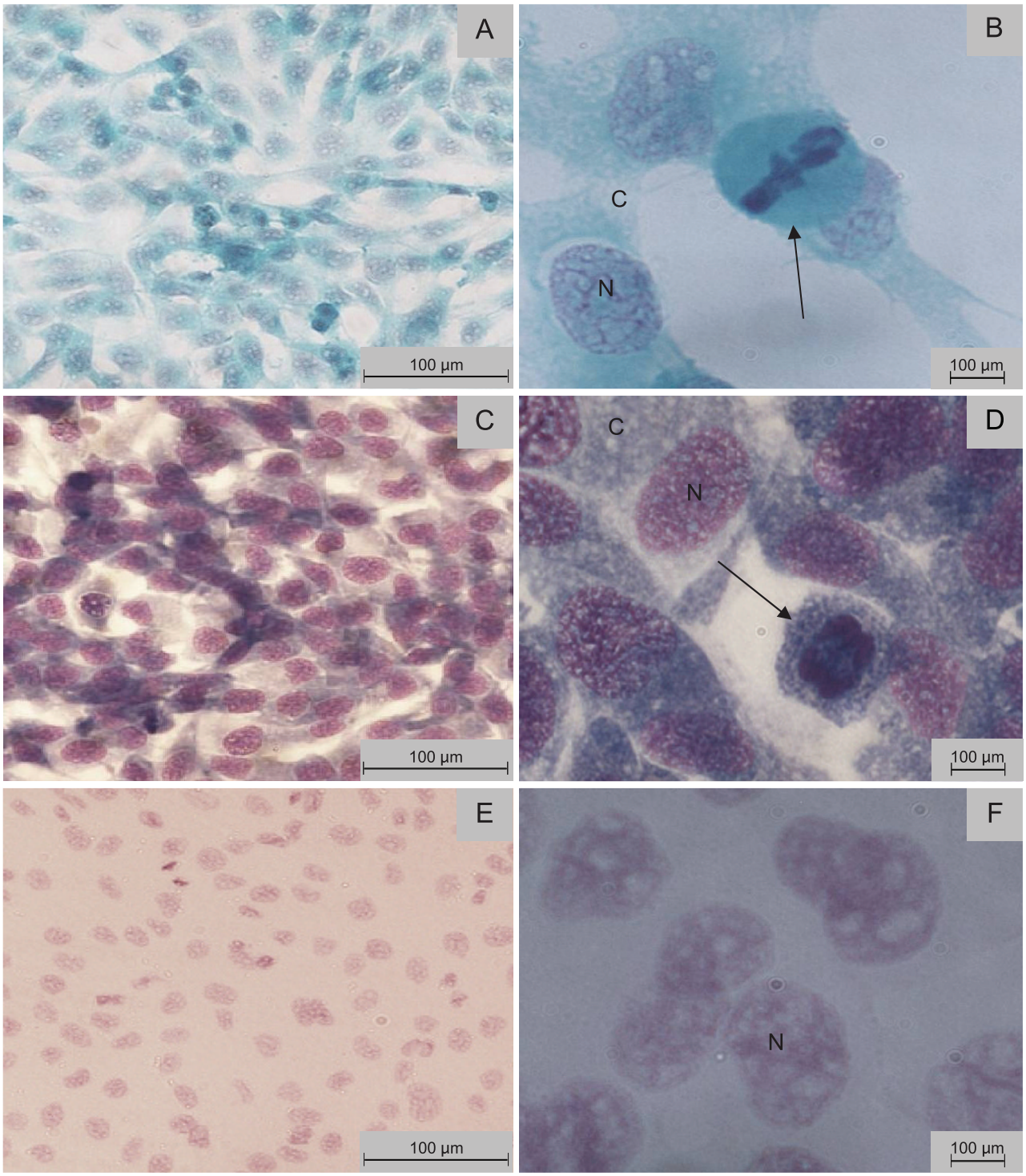

Figure 1. Cells of Chinese hamster lung fibroblasts. (A) Stained with Shiff's and Light Green dyes, fixed with methanol (a cell culture view). (B) Stained with Shiff's and Light Green dyes, fixed with methanol. N-nucleus stained pink, C-cytoplasm stained green, arrow indicates the cell during the mitotic division. (C) Stained with Giemsa stain, fixed with 96% ethanol (a cell culture view). (D) Stained with Giemsa, fixed with 96% ethanol. N-nucleus stained purple, C-cytoplasm stained blue, arrow indicates the cell during mitotic division. (E) Stained with Shiff's dye, fixed with 96% ethanol (a cell culture view). (F) Stained with Shiff's dye and fixed with 96% ethanol. N-nucleus stained pink (Krzyżewski).

The best method of cell staining proved to be the Schiff method with Light Green (Figure 1). The boundaries between cells were well-marked and there was a large contrast between the cytoplasm (stained in green) and the nucleus (stained in pink). The method is rather complicated and time-consuming and allows only for short-term storage of microscope slides (when they are kept in the dark, they can lose contrast after about one year).

In terms of durability, Giemsa staining proved to be the best method (Figure 1). Preparations kept in a dark place maintained their contrast for at least two years. This method is simple and does not require large expenditures of time.

The least effective method of staining was Schiff's staining (Figure 1). The preparations were stained for nuclei only and this method does not provide information on the distribution or cell boundaries.

To summarize: for rapid analysis, Schiff's reagent staining with Light Green colouring is the most appropriate method. However, if long-term cataloguing is necessary, the best choice would be the Giemsa method.

CONCLUSIONS

1. The Schiff method of colouring with Light Green showed the greatest contrast between the nucleus and the cytoplasm; however, the staining was transient and was sensitive to light radiation.
2. The Giemsa staining was the simplest and quickest method and the preparations were suitable for microscopic evaluation; however, the differences in colours of the nucleus and the cytoplasm were not always clearly recognisable.
3. Although the Schiff method visualized the nucleus, it did not provide information on the cytoplasm.

REFERENCES

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