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บทวิทยาการ

ผลของสารคอลชิซีนและไซคลอฟอสฟามายด์ในการเกิดไมโครนิวเคลียสต่อเซลล์เพาะเลี้ยง KB

Micronuclei detection in KB cell lines after exposure to colchicine and cyclophosphamide

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Abstract

Detection of micronuclei has been used to monitor genotoxicity in mammalian cells. Micronuclei are chromatin-containing structures in the cytoplasm produced by exclusion of whole chromosomes or chromatin fragments during cell division. The micronuclei numbers are found to be increased after exposure to genotoxic agents. Epidemiological studies showed micronuclei in human oral cells in subjects previously exposed to genotoxic agents. However, direct *in vitro* micronuclei detection in oral cell lines treated with suspect agents has never been reported. Therefore, the objective of this study was the detection of micronucleus in epidermal carcinoma KB cell lines after exposure to 2 genotoxic agents, colchicine and cyclophosphamide. The KB cell lines were plated on cover slips in a 6-well plate. Various doses (1, 5 and 10 μg) of colchicine and cyclophosphamide (50, 100 and 200 μg) were used to treat KB cells for 5 mins, 1hr and 24 hrs. The specimens were stained and the micronucleated cells counted. In colchicine treated groups, 5-62 cells from 1000 cells were positive for micronuclei, whereas 1-5 cells from 1000 cells of the control groups were observed to have micronuclei. Cyclophosphamide was found to be highly toxic to KB cell lines. Most of the cells were dead, and very low numbers of micronuclei were detected. We suggest this method to be applied for *in vitro* genotoxicity study in oral cells using colchicine as a positive control.

Key Words : Micronuclues, colchicine, cyclophosphamide, KB cell

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บทคัดย่อ

การตรวจสอบสารที่เป็นพิษต่อพันธุกรรมโดยใช้เทคนิคการเกิดไมโครนิวเคลียสต่อเซลล์ของสัตว์เลี้ยงลูกด้วยนมเป็นตัวบ่งชี้ที่มีมานาน ไมโครนิวเคลียสเป็นโครโมโซมส่วนหนึ่งที่หลุดออกมาอยู่นอกนิวเคลียสในขณะที่เซลล์มีการแบ่งตัว ซึ่งจะเพิ่มจำนวนมากขึ้นเมื่อเซลล์ได้รับสารพิษ การศึกษาทางระบาดวิทยาพบว่าเซลล์ในช่องปากมีไมโครนิวเคลียสเกิดขึ้นเมื่อได้รับสารพิษเช่นกัน แต่ยังไม่ได้มีการศึกษาโดยตรงในเซลล์เพาะเลี้ยงที่เกี่ยวข้องกับเซลล์ในช่องปากต่อสารที่สงสัย ดังนั้นในการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาถึงการเกิดไมโครนิวเคลียสในสารพิษ 2 ชนิด คือ สารคอลชิซีน และสารไซคลอฟอสฟามายด์ต่อเซลล์เพาะเลี้ยง KB เซลล์ KB ถูกเลี้ยงในจานเพาะเลี้ยงชนิด 6 หลุม และถูกทดสอบด้วยสารพิษทั้ง 2 ชนิดที่ความเข้มข้นและเวลาที่ต่างๆ กันในสารพิษทั้ง 2 ชนิด คือ 1, 5 และ10 ไมโครกรัมในสารคอลชิซีน และ 50, 100 และ 200 ไมโครกรัมในสารไซคลอฟอสฟามาย ด้วยเวลา 5 นาที, 1 และ 24 ชั่วโมง จากนั้นจึงย้อมสีเพื่อนับจำนวนเซลล์ที่เกิดไมโครนิวเคลียส ผลจากการทดลองพบว่า สารคอลชิซีนทำให้เซลล์ KB เกิดไมโครนิวเคลียส 5-62 เซลล์ต่อ 1000 เซลล์ ในขณะที่กลุ่มควบคุมมีเพียง 1-15 เซลล์ต่อ 1000 เซลล์ ส่วนไซคลอฟอสฟามายพบว่ามีความเป็นพิษต่อเซลล์จนทำให้เกิดการตายของเซลล์มากกว่าการเกิดไมโครนิวเคลียส จึงสรุปว่าสารคอลชิซีนน่าใช้เป็นกลุ่มควบคุมด้านเป็นพิษได้ในการศึกษาสารพิษในช่องปาก

คำหัส : ไมโครนิวเคลียส คอลชิซีน ไซคลอฟอสฟามาย เซลล์เพาะเลี้ยง KB

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Micronuclei detection in KB cell lines after exposure to colchicine and cyclophosphamide

Introduction

The detection of micronuclei has been used to monitor genotoxicity in many mammalian cells such as normal rat-kidney fibroblast cell lines[1], Chinese hamster ovary cells[2], or Chinese hamster lung cell lines[3,4]. This method offered a faster, simpler and less expensive alternative to the metaphase analysis.

Micronuclei (MN) are defined as microscopically visible, round or oval cytoplasmic chromatin[5]. They originate from acentric chromatid, chromosome fragments or whole chromosomes which are not included in the main daughter nuclei following nuclear division. They are the result of chromosome damage. Assessment of the number of micronuclei might be used as a strategy to identify the genotoxic damage in the animal or human cells exposed to the mutagens or carcinogens[6]. The key advantages of the micronuclei assay are the relative ease of scoring and the statistical power obtained from scoring larger numbers of the cells than that typically used in the metaphase analysis[7].

Previous epidemiological studies showed micronuclei in human oral cells in subjects previously exposed to genotoxic agents[8-10]. For *in vivo* experiment, an increase in the number of micronuclei in the exfoliated cells indicated an increase of cancer at the site where cells were desquamated from such as the oral cavity[11,12], esophagus, urinary bladder and cervix [13]. Many studies demonstrated that people who had high a risk of oral cancer such as betel nut chewers, tobacco chewers, tobacco smokers and alcohol consumers, had a higher frequency of micronucleated buccal mucosal cells than in the non-user population[10,14-18]. Therefore, the number of micronuclei in the exfoliated buccal mucosal cells might be used as an indicator to monitor or to predict the efficacy of cancer intervention strategies.

However, direct *in vitro* micronuclei detection in oral cell lines treated with suspect agents have never been reported. In this study, we chose the KB cell line to represent oral cells instead of normal oral keratinocyte due to its simple culture condition. The KB cell was originally thought to be derived from an epidermal carcinoma of the mouth. The KB line has been used extensively in studies of cell nutrition and metabolism, cancer chemotherapy screening, tumorigenicity, and viruses[19]. KB cells have been reported to contain human papilloma virus 18 (HPV-18) sequences[19]. These lines represent an important resource for future studies of the biology of human oral cell lines. Therefore, the aim of this paper was to detect the *in vitro* micronuclei in epidermal carcinoma KB cell lines after exposure to colchicine and cyclophosphamide and to prepare guidelines for their use.

Materials and Methods

1. Reagents

The Colchicine and Cyclophosphamide were from Sigma Chemical Company, USA. Dulbecco's modified Eagles's medium (DMEM)

and fetal calf serum were from Invitrogen, USA. The Penicillin G and streptomycin were from the Government Pharmaceutical Organization, Thailand. The Trypsin was from DIFCO, USA. Basic fuchsin (Fuschin), sodium metasulfite, hydrocholic acid and methanol were from Merck Dermstadt, Germany.

2. Culture cells

KB cells (ATCC No. CCL-17) were obtained from American Type Culture Collection (ATCC)[19]. The cells were cultivated in DMEM supplemented with 10% fetal calf serum, 100 IU penicillin G, and 100 μg/ml streptomycin (10% FCS-DMEM). The cultures were maintained in a 5% CO_2 humidified incubator at 37°C. The cells were subcultured by being trypsinized with 0.05% trypsin in phosphate buffered saline (PBS)[3]. The number of the viable cells was determined by staining with trypan blue for standardizing the amount of cultured-viable cells for each plate.

3. Micronuclei detection

KB ($2x10^5$ cells) were plated on 20 mm cover slips in 6-well plates overnight. The medium was then discarded and cells were exposed to 1 ml of 1, 5 and 10 μg of colchicine or 50, 100 and 200 μg of cyclophosphamide in the serum-free DMEM (SF-DMEM) for 5 mins, 1hr and 24 hrs. One ml of serum-free DMEM was used as a negative control and collected at each time point. Cells were then washed twice with the SF-DMEM and 1ml of 10% FCS-DMEM was added. All plates were further incubated for 1 day and examined under microscope for the assessment of the morphology of the cells. Then, the cover slip with cells from each well was removed, air-dried, fixed in 80% methanol for 24 hrs and allowed to air-dry[17,18]. The cells on the cover slips were stained with the Feulgen reaction, counterstained with Fast green and mounted with permount on the clean glass slides[20].

4. Statistical analysis

The experiment was repeated at least twice for each test compound, with duplicates for each data point. All slides were coded prior to scoring to avoid bias. At least 4000 cells with visible cytoplasm were scored for each treatment. The criteria for identifying micronucleus were based on those given by Countryman and Heddleet[21]. The results of the micronuclei detection were shown as mean ± SD. The significance of the difference between control and treatment groups were statistically analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test.

Results

Pilot experiments were performed initially on all the test solutions to estimate appropriate concentrations (1, 5 and 10 μg/ml for colchicine and 50, 100 and 200 μg/ml for cyclophosphamide) and times (5 mins, 1 hr and 24 hrs) for the experiments involving micronuclei formation. As shown in table 1 for the 5-mins treatment, most cells in all groups contained 1 MN figure 1 and only some cells showed 2, 3 or more MN figure 2. Proportions of cells containing MN (per 1,000 cells) were compared between concentrations and times as summarized in *table 2* and represented in graphic format in *figure 3*. It was found that colchicine at the concentrations of 1, 5 and 10 μg/ml for 5 mins significantly induced an increased number of MN when compared to the negative control and cyclophosphamide. The highest numbers of cells

Table 1 Effect of Colchicine and Cyclophosphamide on frequency of micronucleated cells in KB cell line in 5 min

Treatment	Conc.	Number of MN cells / 1000 cells (mean±SD.)			
	(mg/ml)	1 MN	2 MN	3 MN or more	Total
Control	DMEM	3.50±0.71	1.50±0.71	0	5.00±1.41
Colchicine	1	34.00±2.83	2.00±2.83	3.00±4.24	39.00±1.41*
	5	33.00±7.07	13.00±1.41	16.00±2.83	62.00±5.66*
	10	17.00±15.60	2.00±0	19.00±12.70	38.00±2.83*
Cyclophos	50	5.00±1.41	0	0	5.00±1.41
phamide	100	15.00±7.07	0	0	15.00±7.07
	200	6.00±5.66	0	0	6.00±5.66

*p-value<0.05 significant different from control and cyclophosphamide

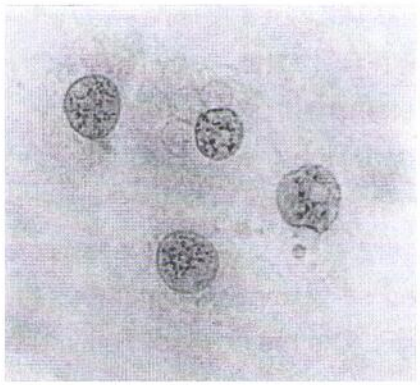

Fig. 1 The KB cell contain one micronucleus

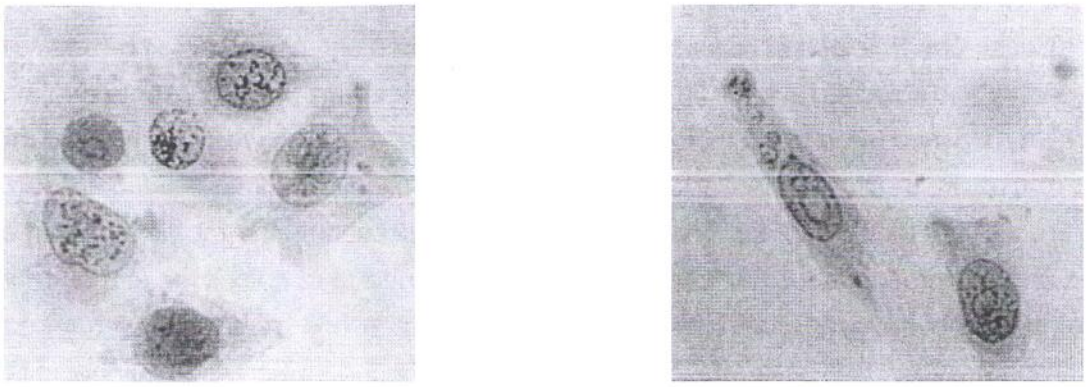

Fig. 2 The KB cell contain 2, 3 or more micronucleus

Table 2 Effect of Colchicine and Cyclophosphamide on frequency of micronucleated cells in KB cell line in various times (Total)

Treatment	Conc.	Number of MN cells / 1000 cells (mean±SD.)		
	(mg/ml)	5 min	1 hour	24 hours
Control	DMEM	5.00±1.41	10.50±7.78	8.50±4.95
Colchicine	1	39.00±1.41*	22.50±6.36	12.00±5.66
	5	62.00±5.66*	38.00±2.83	9.00±4.24
	10	38.00±2.83*	30.50±4.95	17.00±1.41
Cyclophos	50	5.00±1.41	8.50±3.54	14.00±0
phamide	100	15.00±7.07	1.50±2.12	13.00±4.24
	200	6.00±5.66	3.00±2.83	7.00±1.41

*p-value<0.05 significant different from control and cyclophosphamide

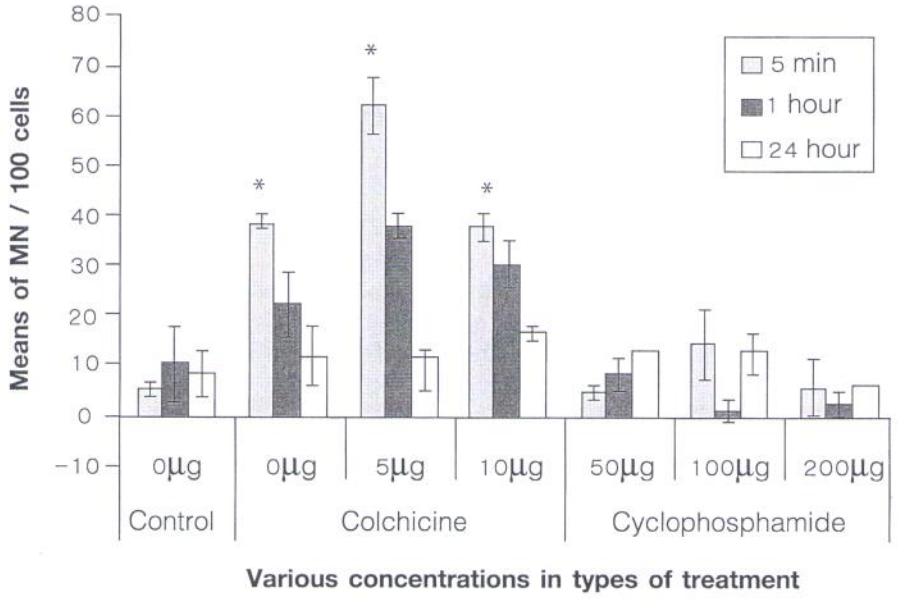

Fig. 3 *Various in the frequencies of MN in KB cells exposed to various concentration of colchicine and cyclophosphamide solution compare with control (*p-value<0.05 significent different from control and cyclophosphamide)*

containing MN was found in 5 µg/ml of colchicine for 5 mins, whereas 1 hr and 24 hrs showed no difference. Strong cytotoxicity was seen in all concentrations of cyclophosphamide where most of the cells were dead. There was no significant difference of MN in the survival cells treated with cyclophosphamide.

Discussion

Colchicine has a cytogenetic effect by inhibition of in vitro microtubule assembly. When spindle microtubules are disturbed during cell division, aneuploidy follows. Another mutagen cyclophosphamide induces DNA breakage, which results in clastogenesis[22]. Liu et al.

studied the effect of colchicine and cyclophosphamide on Chinese hamster lung (V79) cells. They reported that colchicine induced multinucleated cells rather than MN, whereas cyclophosphamide mainly produced MN rather than multinucleated cells[3]. In contrast, our present study on KB cells reveals that colchicine has a stronger effect than cyclophosphamide on MN induction. The difference might be partly due to the cell type, in which we used KB because of its epithelial origin[19]. KB cells have been reported to contain human papilloma virus 18 (HPV-18) sequence[19] which produced E6 and E7 oncoprotein. Both proteins were found to be able to induce multinucleation[23-27] but no study had been done on micronucleus. Another difference might be the need for using S9 in combination with cyclophosphamide. S9 is a rat liver microsome mixture. It is used as a simultaneity for cyclophosphamide in many studies[3,4]. In this study we omitted S9 since we tried to provide similar conditions between the test and control group. As a result, even though we tried various concentrations prior to this experiment, cyclophosphamide without S9 is not effective in inducing MN. Further, it was found to be highly toxic as most of the cells were dead.

In daily life, oral cavities are exposed to various carcinogens from food, drinks, dentrifice, mouthwashes, etc. The use of 5μg/ml colchicines for 5 minutes can be useful as a positive control for testing those suspected agents, and the 5-minute exposure time seems reasonable for most agents.

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