The *in vitro* Micronucleus Assay for Cigarette Smoke Condensate Samples: Photomicrographs for Micronucleus Scoring and Analysis of Historical Data Obtained from Kentucky Reference Cigarette 3R4F

SAWAGURI J.[1] ; HORI K.[2] ; SAKIMURA M.[3] and SOFUNI T.[1]

[1] Japan Tobacco INC., Scientific and Regulatory Affairs Division, [2] Mitsubishi Chemical Medience CORP., Drug Development Service Segment, [3] Japan Tobacco INC., Product Science Division

Introduction

The *in vitro* micronucleus (MNvit) assay is a genotoxicity test that was developed as an alternative to the chromosomal aberration assay with the advantage to detect both structural aberrations and numerical aberrations (aneuploidy). In addition, MNvit assay can be conducted rapidly and easily compared with the chromosomal aberration test with the better statistical power and the possibility of automation.

We have been conducted the MNvit assays for the evaluation of cigarette smoke condensate (CSC) samples to detect the genotoxicity of tobacco materials and/or ingredients as one of the in vitro toxicity assay. In order to maintain the uniformity of the data evaluation, we prepared photomicrographs of micronucleated cells to define detailed micronucleus (MN) classifications. The accumulated historical data of the micronuclei (MNi) in the CSC samples derived from 3R4F was analyzed from several aspects.

Materials & Methods

CSC Collection
- Cigarette: Kentucky Reference Cigarette 3R4F
- Smoking condition: ISO 3308 and ISO 4387
- Smoking machine: RM200 (Borgwaldt)
- Filter pad: φ44 mm glass fiber filter pad (Borgwaldt)
- Solvent: DMSO (final concentration in the culture medium: 2%)

Culture condition
- Cell line: CHL/IU
- Medium: Eagle's MEM with 10% (v/v) heat-inactivated bovine serum
- Cell seeding: 5.2 × 10^4 cells/mL
- Pre-culture: 24 h

Treatment condition
- Plate: 60 mm diameter
- Treatment schedule: 3 h treatment – 21 h recovery
- Actin polymerization inhibitor: none (without cytochalasin B)
- Metabolic activation: with or without S9
- Test system for S9 preparation: rats
- S9 inducer: Phenobarbital and 5,6-benzoflavone
- S9 concentration in the culture medium: 1.5%
- Replicate: duplicate
- No. of cells counted: 1000 cells/plate, 2 plates/dose (2000 cells/dose in total)
- Cytotoxicity assessment: relative cell count (RCC measure including the data showed >60% cytotoxicity)

Target cells for observation
- The cells with a normally stained nucleus (round or oval) and clear cytoplasm were targeted for MN observation. The cells which were weakly/abnormally stained or those thought to be pyknosis or apoptosis were excluded from the observation.

MN classification [1)]
- MN-1: Cell with one or more MNi with diameters not exceeding 1/10 the diameter of the main nucleus.
- MN-2: Cell with one MN with diameter greater than 1/10 but not exceeding 1/3 the diameter of the main nucleus.
- MN-3: Cell with one MN with diameter greater than 1/3 but not exceeding 1/2 the diameter of the main nucleus.
- Mu-MN: Cell with multiple MNi with diameters greater than 1/10 but not exceeding 1/2 the diameter of the main nucleus.
- MN-T: Total of MN-2, MN-3 and Mu-MN

Note: If there is a borderline-sized MN, then brightness, shape and size of the MN are allowed to determine the classification (especially distinction between MN-1 and MN-2).

Normal Cell		MN-2	
MN-1		Mu-MN	
MN-2		Mu-MN	
MN-3		MN-2	
MN-1		Mu-MN	

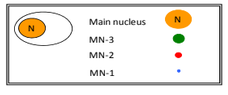

Fig. 1 Images of MN

1) Matsushima, T., Hayashi, M., Matsuoka, A., Ishidate, M., Jr., Miura, K.F., Shimizu, H., Suzuki, Y., Morimoto, K., Ogura, H., Mure, K., Koshi, K. and Sofuni, T. (1999) Mutagenesis, 14, 569-580.

Results & Discussion

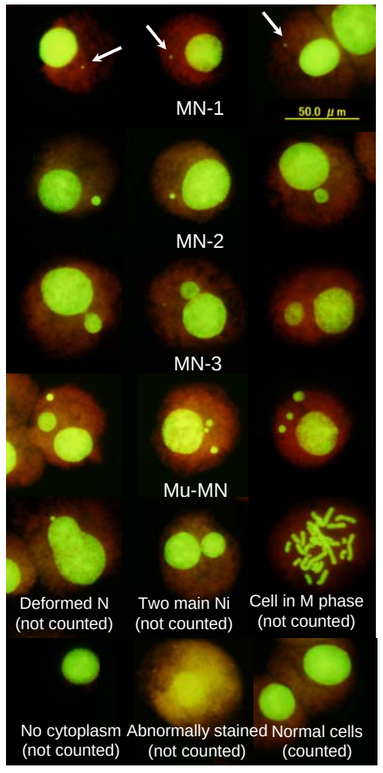

Fig. 2 Typical photomicrographs of MNi

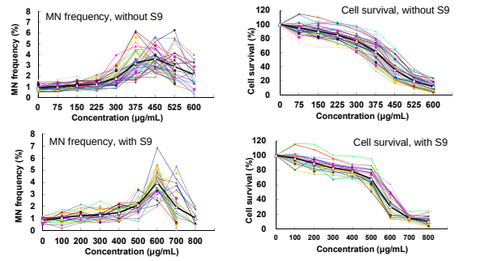

Fig. 3 Historical data of MN frequency and cell survival

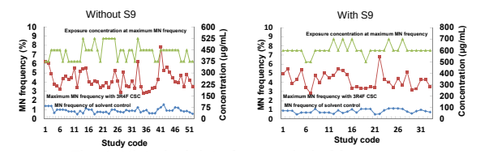

Fig. 4 Historical data for a period of few years

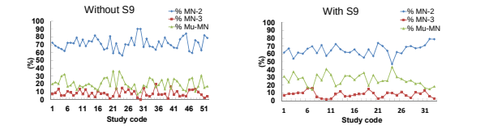

Fig. 5 Percentages of MN-2, MN-3 and Mu-MN in MN-T at a dose showed maximum MN frequency

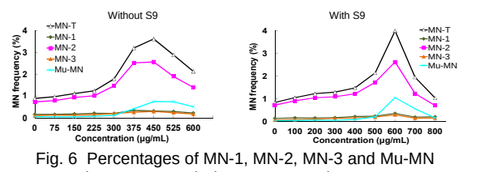

Fig. 6 Percentages of MN-1, MN-2, MN-3 and Mu-MN in MN-T at each dose concentration

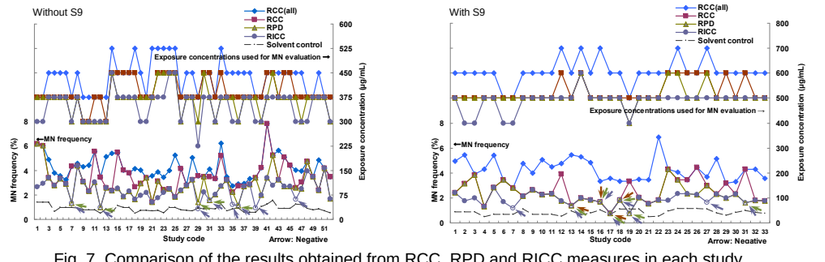

Fig. 7 Comparison of the results obtained from RCC, RPD and RICC measures in each study

RCC(all): RCC measure including the data showed >60% cytotoxicity

Individual and Average Historical Data

The MN frequency and cell survival are shown in Fig. 3 (thin lines: individual values, heavy black lines: mean values).

Mean MN frequency increased with increasing dose and the maximum MN frequencies were found to be 450 μg/mL without S9 mix and 600 μg/mL with S9 mix. Cell survival decreased with increasing dose, and 50% cytotoxicity was produced between 375 and 450 μg/mL without S9 mix and between 500 and 600 μg/mL with S9 mix. In the presence of S9 mix, mean MN frequency peaked sharply at 600 μg/mL and cell survival decreased severely between 500 and 600 μg/mL compared with that in the absence of S9 mix. No clear explanation can be given for these differences between with and without S9 mix, but it was suggested that the toxicological profile might have been altered by enzymes in S9.

Transition of Historical Data

Transition of historical data in individual studies is shown in Fig. 4.

In the absence of S9 mix, MN frequency of the solvent control ranged from 0.5 to ~1.5% and the maximum frequency of MN in the CSC sample treated groups ranged from ~3 to ~8%. The dose concentration at the peak MN frequency ranged 375–525 μg/mL.

In the presence of S9 mix, MN frequency of the solvent control ranged from 0.5 to ~1.0% and the maximum frequency of MN in the CSC sample treated groups ranged from ~3 to ~7%. The dose concentration at the peak MN frequency ranged 500–700 μg/mL.

Overall, the variability of MN frequencies over time indicated that the data did not vary significantly during the period.

Composition of MN-T in Each Study or Each Dose Concentration

The contents of MN-T (percentage of MN-2, MN-3 and Mu-MN in MN-T) for individual studies are shown in Fig. 5, and mean frequencies of MN-1, MN-2, MN-3, Mu-MN and MN-T are expressed in Fig. 6.

MN-2 was the most frequent in MN-T, followed by Mu-MN and MN-3 in most of the studies, both with and without S9 mix. This proportion continued during the period. MN-3 did not apparently increase with increasing dose. MN-1, which was not included in MN-T, showed no clear increase with increasing dose.

Overall, the distribution of MN-T (MN-2, MN-3 and Mu-MN) has been maintained stably over time and most of the components in MN-T was MN-2 and Mu-MN in MNvit assays of the CSC sample.

Positive/negative results obtained from RCC, RPD and RICC measures

The difference of positive/negative results between RCC, RPD and RICC measures is expressed in Fig. 7. The RCC measure was calculated in two different ways: one includes the data that showed >60% cytotoxicity [RCC(all)], and the other does not include the data that showed >60% cytotoxicity (RCC). The RPD and RICC measures do not include the data that showed >60% cytotoxicity.

In the absence of S9 mix, the exposure concentrations at the highest MN frequency by RCC(all), RCC, RPD and RICC measures ranged 375–525 μg/mL, 300–450 μg/mL, 300–450 μg/mL and 225–450 μg/mL, respectively. The highest MN frequencies by RCC(all), RCC, RPD and RICC measures were ranged 2.75–7.85%, 1.40–7.85%, 1.00–6.20% and 0.85–4.40%, respectively. Although RCC(all) and RCC gave no negative response on these 52 occasions, RPD and RICC measures gave negative responses on 5/52 occasions (green arrows) and 8/52 occasions (purple arrows), respectively.

In the presence of S9 mix, the exposure concentrations at the highest MN frequency by RCC(all), RCC, RPD and RICC measures were ranged 500–700 μg/mL, 500–600 μg/mL, 400–600 μg/mL and 400–600 μg/mL, respectively. The highest MN frequencies by RCC(all), RCC, RPD and RICC measures ranged 2.80–6.85%, 0.75–4.45%, 0.75–4.30% and 0.75–2.85%, respectively. Although RCC(all) gave no negative response on these 33 occasions, RCC, RPD and RICC measures gave negative responses on 4/33 occasions (brown arrows), 6/33 occasions (green arrows) and 8/33 occasions (purple arrows), respectively.

Conclusion

The OECD Test Guideline 487 (MNvit assay) states that when cytochalasin B is not used, evaluation of cytotoxicity based on RICC or on RPD is recommended and the highest concentration should aim to produce 55±5% cytotoxicity. CSC samples can be evaluated with these criteria, but CSC samples are sporadically judged to be "negative" when RICC or RPD are applied.
We assume that CSC samples fundamentally increase MN frequency and therefore the comparison of potential toxicity of two or more CSC samples is more important than positive/negative judgment. In other words, since we use MNvit assay for evaluation of the difference between cigarette samples with additives and those without additives, the data with more than 60% cytotoxicity should be included in the evaluation. Therefore, we decided to use RCC for cell survival and to include the data with more than 60% cytotoxicity.