Short communication

Study on the *in vivo* Comet Assay Using Rat Uteri

Izumi Ogawa¹, Satoshi Furukawa, Yoshinori Tanaka, Masayoshi Abe,
Koji Usuda, Seigo Hayashi and Hiroshi Onitsuka

Biological Research Laboratories, Nissan Chemical Industries, Ltd., Saitama, Japan

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In order to examine the utility of the *in vivo* Comet assay for evaluating genotoxicity in the uterus, we performed the Comet assay using rats' livers and uteri for four compounds: methyl methanesulfonate (MMS) and *N*-nitroso-*N*-diethylamine (DEN) as genotoxic compounds, indole-3-carbinol (I3C) and diethylstilbestrol (DES) as non-genotoxic compounds. Whether or not the sexual cycles affected the outcome of this assay was also investigated by treatment with saline and MMS. The results showed that there was no statistically significant difference in tail moment among the three sexual cycles either with the saline or MMS treatment. MMS and DEN, genotoxic carcinogens, induced a significant increase in the tail moment in both the uterus and liver. I3C, a non-genotoxic non-carcinogen, and DES, a non-genotoxic carcinogen, did not increase the tail moment significantly in either organ. We confirmed that the Comet assay using rat uteri can be used without considering the sexual cycle to assess *in vivo* genotoxicity in the uterus and help clarify the mechanism of carcinogenesis.

Key words: *in vivo* Comet assay, uterus

Introduction

The *in vivo* Comet assay is being increasingly used to evaluate the *in vivo* genotoxic potential and mechanism or cause of carcinogenicity (1). One of the advantages of this assay is that it can be conducted for various organs, irrespective of the cell proliferation. This means that the assay can contribute to elucidating the mechanism of the carcinogenesis generated in the uterus. The uterus, affected by sexual hormones, is one of the tumor target sites for female. Among such hormones, 17 β -estradiol is considered to induce carcinogenesis by hormone receptor-mediated proliferation of cells whose DNA is damaged by the metabolite of this hormone (2, 3). Therefore, when the incidence of uterine carcinogenesis is found to be due to developing agrochemicals, food additives and pharmaceuticals, it is important to evaluate the DNA damaging potential of the candidate chemicals to clarify the mechanism. Although there have been many reports about performing this assay using multiple organs, such as the stomach, colon, kidneys, urinary bladder, lungs, brain, bone marrow and liver to

date, those with the uterus are limited (4–6). To investigate the efficacy and methodological factors for using the uterus in the Comet assay, we examined whether or not the three sexual cycles, proestrus, metestrus and anestrus, in which hormonal levels are diversified, would affect the outcome of the assay and then validated the reactivity to genotoxic and non-genotoxic compounds. To compare the effect of the sexual cycles, methyl methanesulfonate (MMS) was administered to rats divided into the three different sexual cycles. To investigate the reactivity, MMS and *N*-nitroso-*N*-diethylamine (DEN) were used as genotoxic compounds and diethylstilbestrol (DES) and indole-3-carbinol (I3C) as non-genotoxic compounds.

Materials and Methods

Chemicals: MMS (66–27–3), regular (GP-42) agarose and low melting point (LGT) agarose were purchased from Nacalai Tesque Co., Ltd., (Kyoto, Japan). DEN (55–18–5) was purchased from Tokyo Kasei (Tokyo Japan). DES (56–53–1) and I3C (700–06–1) were purchased from Sigma-Aldrich Chemicals Inc. (St. Louis, USA).

Experiment 1: A total of 18 specific pathogen-free BrlHan:WIST@Jcl (GALAS) female rats purchased from CLEA Japan, Inc., approximately 19 weeks of age, were kept in hanging stainless steel mesh cages individually during the experimental period. The room was air-conditioned (temperature, $22 \pm 2^\circ\text{C}$; humidity, $55 \pm 10\%$; light cycle, 12 hr/day), and food (Oriental Yeast Co., LTD., Japan) and water were available *ad libitum*. The sexual cycles of all animals were observed by the vaginal smear method for about a week before treatment (7). Animals were assigned into three groups according to sexual cycles, proestrus (I), metestrus (III) and anestrus (IV), each of which was further divided into two groups: one was orally treated with MMS at 160 mg/kg (1 mL/100 g body weight of rats), and the

¹Correspondence to: Izumi Ogawa, Biological Research Laboratories, Nissan Chemical Industries, Ltd., 1470, Shiraoka, Minamisaitama, Saitama 349-0294, Japan. Tel: +81-480-92-2513, Fax: +81-480-92-2516, E-mail: ogawai@nissanchem.co.jp

other was orally treated with saline as a negative control. Three hours after treatment, animals were sacrificed by cervical dislocation after exposure to rising levels of carbon dioxide. After removal, the uterus was cut open and gently stretched out sideways, then homogenized using a Potter-type homogenizer. All procedures used to prepare agar slides were the same as those described previously (8). The slides were placed on a horizontal gel electrophoresis platform and covered with a chilled alkaline solution (pH > 13) and left in the dark at 0°C for 10 minutes. The nuclei underwent electrophoresis for 15 min at 1 v/cm (250–300 mA).

The Comet images were scanned into an image analyzer (SCG32; Keio denshikogyo Co., Japan) to measure the tail moments. The tail moment was obtained by observing 50 nuclei for each animal. The average tail moment of the MMS treatment group was compared to that of the negative control group in the same sexual cycle, and the averages of all nine animals in the MMS treatment and the negative control group were also compared without considering sexual cycles. Those groups were checked for their homogeneity of variance using the F-test. If homogeneity was found, a comparison was performed using the Student-*t* test, if not, using the Aspin-Welch test. The tail moments for each sexual group for the same treatment were compared using Tukey's multiple comparison test to identify heterogeneous responses of sexual cycles on tail moments.

Experiment 2: A total of 48 specific pathogen-free BrlHan:WIST@Jcl (GALAS) female rats purchased from CLEA Japan, Inc., approximately eight weeks of age, were kept in the same way as in experiment 1. The animals were randomly assigned without considering their sexual cycles into exposure groups of four rats each. MMS and DEN were treated orally at 160 mg/kg and 320 mg/kg (1 mL/100 g body weight of rats), respectively, on the ground that positive responses were reported to be found in the stomach, colon, kidneys, urinary bladder, lungs, brain, bone marrow and liver for MMS, and also all those organs except bone marrow for DEN in these conditions (6, 9, 10). I3C was administered orally at 2000 mg/kg (1 mL/100 g body weight of rats), the maximum dosage for a genotoxicity study. DES were treated intraperitoneally at 10 and 20 mg/kg (1 mL/100 g body weight of rats), about half of LD50 (34 mg/kg for intraperitoneal injection). Animals in the negative control group were treated orally with saline. At 3 and 24 h after treatment, the animals were sacrificed in the same way as in experiment 1. After removal, the liver was minced into rectangles and the uterus was cut open and gently stretched out sideways, with all procedures being the same as in experiment 1.

These experiments were conducted according to the Guidelines for Animal Experimentation (Japanese As-

sociation for Laboratory Animal Science, 1987)

Results and Discussion

There were no deaths or clinical signs during the experimental period. There was no change in any organ by macroscopic observation.

Experiment 1: Table 1 shows the average tail moment of the uterus at 3 h after treatment with the vehicle and MMS in each sexual cycle: proestrus, metestrus and anestrus. MMS produced a statistically significant increase in the tail moment of all the treated animals compared with that of all the negative control animals, and furthermore, a statistically significant increase in MMS was also found within each identical sexual cycle. There was no statistically significant difference in the tail moment among the three sexual cycles both for the MMS treatment and the negative control groups. This indicates that rat uteri can be used without considering the sexual cycle when performing the Comet assay.

Experiment 2: Table 2 shows the average tail moments of the liver and uterus after treatment with the four compounds. The negative control value in the uterus was almost twice as much as that in the liver. DEN and MMS produced statistically significant increases in the tail moments both in the uterus and liver. DES and I3C did neither in the uterus nor liver.

DEN: A statistically significant increase in the tail moments was found in the uterus at 3 h after treatment. At 24 h after treatment, they increased to almost as much as at 3 h, although this was not statistically significant. The tail moment of the liver showed a statisti-

Table 1. Tail moment in uteri of rats treated with MMS in various stages of the sexual cycle

MMS (mg/kg)	No. of animal	Sexual cycle*	Tail moment†
0*	3	I	0.83 ± 0.12
	3	III	0.85 ± 0.08
	3	IV	0.80 ± 0.07
	9	I + III + IV	0.83 ± 0.05
160	3	I	2.04 ± 0.22‡
	3	III	1.70 ± 0.27§
	3	IV	1.67 ± 0.19§
	9	I + III + IV	1.80 ± 0.13¶

*: I; proestrus, III; metestrus, IV; anestrus

†: Mean ± SE

‡: Saline for vehicle control

§ and ‡: Significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively (Student-*t* test).

¶: Significantly different from the control at $p < 0.01$ (Aspin-Welch-*t* test).

In the same treatment, no heterogeneous responses of sexual cycle on the tail moment (Tukey's multiple comparison test) were observed. Saline and MMS were administered orally.

Table 2. Tail moment in the livers and uteri of rats treated with MMS, DEN, DES and I3C

Compound	Dosage (mg/kg)	No. of animal	Tail moment*			
			Liver		Uterus	
			3 h	24 h	3 h	24 h
None†		4	0.23 ± 0.03	0.27 ± 0.04	0.42 ± 0.06	0.49 ± 0.03
MMS	160	4	0.98 ± 0.16	0.31 ± 0.04	1.09 ± 0.07 [‡]	0.77 ± 0.09
DEN	320	4	0.72 ± 0.03 [‡]	2.46 ± 0.31 [‡]	0.76 ± 0.10 [‡]	0.78 ± 0.18
DES	10	4	0.27 ± 0.03	0.26 ± 0.02	0.28 ± 0.03	0.53 ± 0.05
	20	4	0.27 ± 0.02	0.25 ± 0.03	0.39 ± 0.02	0.43 ± 0.02
I3C	2000	4	0.24 ± 0.03	0.24 ± 0.03	0.40 ± 0.08	0.59 ± 0.05

*: Mean ± SE

†: Saline for vehicle control

‡ and §: Significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively (Student-*t* test).||: Significantly different from the control at $p < 0.05$ (Aspin-Welch-*t* test).

Saline, MMS, DEN and I3C were administered orally, and DES was intraperitoneally.

cally significant increase both at 3 h and 24 h after treatment. The value at 24 h was three times as much as that at 3 h. These results suggest that the DNA damage in the uterus was induced by a metabolite of DEN that was produced in the liver. DEN induces carcinogenesis in the stomach, kidneys, lungs and liver of rats (11). The positive responses in the uterus are attributed to the *in vivo* genotoxic potential which was reported to be found in four other organs (colon, urinary bladder, brain, bone marrow), although they are not tumor targets (6, 10).

MMS: The tail moment of the uterus showed a statistically significant increase both at 3 h and 24 h after treatment. On the other hand, statistically significant increase was found only at 3 h after treatment in the liver. This may indicate that MMS remained in the uterus and continued to damage DNA, or that the damage was not repaired, although MMS was rapidly detoxified or repaired in the liver within 24 h after treatment. MMS is reported to induce carcinogenesis in the hematopoietic system and lungs of mice, but there are no data about rats (11). The positive responses in the uterus and liver may be attributed to the *in vivo* genotoxic potential, which was reported to be found in seven other organs as well (9, 6).

DES: DES, formerly used as the synthetic estrogen, induced no DNA damage either in the liver or uterus in this study. It is known to induce tumors in multiple organs of rats/mice, including the uterus in mice (11). There have been reports of negative results on gene mutation tests for bacteria and V79 cells, and of no induction of chromosome structural aberrations, although there were numerical aberrations in the *in vitro* cytogenetic test (12–14). Although marginal positive responses have been reported for the *in vivo* micronucleus test, a negative result was reported for the *in vivo* Comet assay performed with eight organs of male mice (6, 15–17). Moreover, several contradictory

results were found in *in vitro* and *in vivo* assays for detecting DNA adducts (18, 19). The result of our study using rat uteri also supports the theory of non-genotoxicity for DES.

I3C: I3C, which is contained in cruciferous vegetables is considered to be a non-genotoxic compound because of negative results on the Ames test and *in vitro* cytogenetic tests, although there are no reports on *in vivo* genotoxic tests (20, 21). I3C is reported to promote cancer in the uterus of rats under a particular condition, although, it is known to be an anti-carcinogen (22, 23). As for the mechanism, it is believed that I3C induces CYP 1B1, followed by metabolism of 17- β -estradiol to 4-hydroxyestradiol which is considered to be a genotoxic compound that induces carcinogenesis (22, 24). The absence of DNA damage observed in our study could be attributed to the lack of CYP 1B1 induction by single-dose administration of I3C, and to the fact that I3C itself has no potential for *in vivo* genotoxicity.

The values for the uterus tail moment in experiment 2 were smaller than those in experiment 1. The reason for this remains unclear, although the age difference of the animals between the two experiments is suspected. However, this would not affect the accuracy of the experiment as we used animals of the same age. Our results give additional support to the report that genotoxic compounds induce DNA damage in multiple organs, including tumor sites (25).

In conclusion, the Comet assay using rat uteri can be used without considering the sexual cycle to assess *in vivo* genotoxicity in the uterus and to help clarify the mechanism of carcinogenesis.

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