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Data Report

Comet assay of thyroid gland in rats treated with ethyl methanesulfonate

Naho Tsuji, Soichiro Hagio, Kazuya Takeuchi and Satoshi Furukawa

Biological Research Laboratories, Nissan Chemical Corporation,
1470 Shiraoka-shi Shiraoka, Saitama 349-0294, Japan

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ABSTRACT — *In vivo* comet assays are often used to evaluate genotoxicity. The advantage of an *in vivo* comet assay is that it can be applied to a variety of organs. In general, the liver and stomach are used in an *in vivo* comet assay. However, there have also been some reports on the *in vivo* comet assay of the thyroid gland, which is a common target organ for carcinogens in rodents. Ethyl methanesulfonate (EMS) is listed as a compound likely to be the positive control compound of choice in an *in vivo* comet assay, since it is known to cause DNA damage in a variety of tissues, including the liver, stomach, kidneys, and bone marrow. In contrast, there have been no reports on *in vivo* comet assays of the thyroid gland in rats treated with EMS. In the present study, we examined whether EMS also exhibits DNA damage in the *in vivo* comet assay of the thyroid gland in rats. EMS was administered orally at a dose of 200 mg/kg. The results showed a significant increase in mean % tail DNA in the thyroid gland, the extent of which was similar to that in the liver. Since histopathological examination of the thyroid gland revealed no histological findings, the increase in mean % tail DNA was most likely due to DNA damage rather than tissue damage. These results suggest that EMS can be used as a positive control compound in the *in vivo* comet assay of the thyroid gland in rats.

Key words: Comet assay, Ethyl methanesulfonate, Genotoxicity test, Rat, Thyroid gland

INTRODUCTION

The mechanisms of carcinogenesis are divided roughly into genotoxicity and non-genotoxicity. Since genotoxicity is a serious issue in the development of agrochemicals and pharmaceuticals, it is important to determine whether or not the carcinogenic mechanism is due to genotoxicity. Recently, the *in vivo* comet assay has been widely used as a method for detecting DNA damage. The advantage of an *in vivo* comet assay is that it can be applied to genotoxic evaluation in a variety of organs. In general, *in vivo* comet assays are performed in the liver and stomach. In particular, because the liver is the main organ involved in the metabolic activation of compounds and one of the typical target organs for carcinogenicity, while the stomach is the first site of contact after oral exposure, these are used for evaluation (Sasaki *et al.,* 2000; Uno *et al.*, 2015a, 2015b). In addition, apart from the liver and stomach, there have also been reports of *in vivo* comet assays in rats using the kidneys, urinary bladder, bone marrow, blood, ovaries, testes, and adrenal glands, for the purpose of assessing DNA damage in various target organs, depending on the carcinogenic properties of the compounds (Antonietta *et al.*, 2002; Francesca *et al*., 2005, 2006; James and Lisa, 2005; Kitamoto *et al.*, 2015; Kraynak *et al.*, 2015; Leite *et al*., 2007; OECD, 2016; Uno *et al.*, 2015a, 2015b; Wada *et al*., 2012). The thyroid gland is also one of the major target organs in rat carcinogenic-

Correspondence: Naho Tsuji (E-mail: tsujin@nissanchem.co.jp)

ity studies. Although there are some reports of *in vivo* comet assays of the thyroid gland in rats (Al-Amoudi, 2018; Antonietta *et al.*, 2002; Francesca *et al*., 2005, 2006; James and Lisa, 2005; Leite *et al.*, 2007), very few reports have been conducted with positive control compounds.

A positive control group is recommended to be provided for each test in the conduct of an *in vivo* comet assay, and a positive control compound may be selected if appropriate with justification, and demonstrating clear positive responses in the tissues of interest (OECD, 2016). Although ethyl methanesulfonate (EMS) is listed as a compound likely to be the positive control compound of choice in *in vivo* comet assays (OECD, 2016), there have been no reports of *in vivo* comet assays of the thyroid gland in rats treated with EMS. Therefore, this study evaluated the effects of EMS on the rat thyroid gland in an *in vivo* comet assay, in order to confirm the usefulness of EMS as a positive control compound.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from the following suppliers: methylcellulose (MC; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan); ethyl methanesulfonate (EMS; CAS No., 62-50-0; Kanto Chemical Co., Inc. Tokyo, Japan), which was dissolved in physiological saline (Otsuka Pharmaceutical Factory, Tokushima, Japan); low melting point agarose (NuSieve GTG Agarose: Lonza, MD, USA); and, SYBR Gold (Invitrogen-Molecular Probes, OR, USA).

Animals

Specific pathogen-free RccHan™:WIST male rats, approximately 8 weeks of age, were purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan). The room was air-conditioned (temperature, 22 ± 2ºC; humidity, 55 ± 10%; light cycle, 12 hr/day), and diet (CRF-1: Oriental Yeast Co., Ltd., Tokyo, Japan) and water were available *ad libitum*. Animals were observed for clinical signs and weighed throughout the study. This study was conducted according to the Guidelines for Animal Experimentation of the Biological Research Laboratory at Nissan Chemical Corporation.

Animal treatment

Ten animals were randomly assigned by body weight to two groups of five animals each. The animals in the EMS group were orally treated with EMS at 200 mg/kg (10 mL/kg body weight), according to the *in vivo* Mammalian Alkaline Comet Assay test guideline (OECD, 2016) and the reference papers (Kitamoto *et al.*, 2015; Kraynak *et al.*, 2015; Uno *et al.*, 2015a, 2015b; Wada *et al.*, 2012). The animals in the control group were orally treated with 0.5% MC solution. Once daily for two days, animals were euthanized by carbon dioxide inhalation at 3 hr after the final administration, followed by abdominal exsanguination, and the thyroid gland and liver were removed.

Comet assay method

The *in vivo* comet assay was conducted according to a previously published method, with some modifications (Hagio *et al.*, 2014). For the thyroid gland, one side of the excised thyroid gland was used for the *in vivo* comet assay. To facilitate cell separation, a slit was made in the serous membrane surrounding the thyroid gland. A total of 3 mL of ice-cold homogenizing buffer was added to the thyroid gland, and the mixture was homogenized slowly at 700 rpm in a 10-mL Teflon Potter homogenizer. After homogenization, the solution was passed through a 40 μm cell strainer to prepare a single cell solution. For the liver, hepatocytes were minced from the liver in an ice-cold homogenizing buffer, using shears. Afterwards, the solution was passed through a 40 μm cell strainer to prepare a single cell solution. Each cell-suspension was mixed with 0.5% (wt/vol) low melting point agarose (single cell solution: agarose = 1:9), and the resulting mixture was mounted on MAS coated glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan). After solidification, each slide was placed in cold lysing solution overnight under refrigeration, to lyse the nuclei. After lysing, the slides were electrophoresed in electrophoresis buffer. Electrophoresis was carried out at a constant voltage of 0.7 V/cm for 20 min, under protection from light. After electrophoresis, slides were transferred to neutralizing buffer, and were then dipped in methyl alcohol and dried. Each slide of all animals was stained with SYBR Gold, and observed under a fluorescence microscope. An image analyzer (Comet Assay IV Image Analysis System: Perceptive Instruments, Instem Group of Companies, Staffordshire, UK) was used to calculate the % tail DNA. One hundred and fifty nuclei, excluding hedgehog, from 1 or more slides per animal were randomly observed with a magnification power of 200, and the % tail DNA (also known as % tail intensity) was calculated. The % tail DNA is determined by the DNA fragment intensity in the tail, expressed as a percentage of the cell's total intensity. The median of the % tail DNA was calculated for each slide and the mean of the median % tail DNA was determined for each animal, while the group mean value and standard deviation were calculated for each group. Hedgehog frequency was not measured.

Table 1. Result of comet assay in thyroid grand and liver.

Compound	dose level (mg/kg)	No. of animal	Comet assay % tail DNA - group mean values	
			Thyroid grand	Liver
0.5% MC	0	5	0.46 ± 0.16	0.30 ± 0.10
EMS	200	5	7.00 ± 0.83[##]	5.88 ± 2.20[##]

(mean ± SD)
Abbreviations: MC, methylcellulose; EMS, Eethyl methanesulfonate
[##]: Significantly different from control, $p < 0.01$. (Aspin-Welch test)

Histopathological examination

The sides of the thyroid glands not used in the *in vivo* comet assay were fixed and preserved in 10% neutral formalin buffer. They were embedded in paraffin, and 4 μm thick sections were stained routinely with hematoxylin and eosin for histopathological examination.

Statistical analysis

Statistical analysis was performed on the data of the mean % tail DNA using Pharmaco Basic (Scientist Press Co., Ltd., Tokyo, Japan). For comparisons between the control and EMS groups, the F-test was performed, followed by the Aspin-Welch test.

RESULTS

There were no deaths or clinical signs during the experimental period in each group. The means of the % tail DNA were 0.46 and 7.00 in the thyroid gland and 0.30 and 5.88 in the liver, in the control and EMS groups, respectively. The means of the % tail DNA in both organs showed a statistically significant increase in the EMS group, compared to the control group (Table 1). There were no histopathological changes in the thyroid gland, in either group.

DISCUSSION

In this study, we evaluated whether EMS induces DNA damage in the *in vivo* comet assay of the thyroid gland in rats. Administration of EMS to rats at the published dose, as a positive control in the liver and stomach (Kitamoto *et al*., 2015; Kraynak *et al.*, 2015; Uno *et al*., 2015a) resulted in a significant increase in % tail DNA in the thyroid gland, compared to the control group. The data acceptance criteria in an *in vivo* comet assay validation study (Uno *et al.*, 2015b), in which EMS was used as a positive control compound in the liver and stomach were that % tail DNA must be 1) statistically significantly increased relative to the control, 2) increased by more than 5% relative to the control, and 3) increased by 2-fold or more, relative to the control. The increase in % tail DNA by EMS in this study met all of the above data acceptance criteria for both liver and thyroid gland. Since an increase in % tail DNA must be considered not only for genotoxic, but also cytotoxic effects (OECD, 2016), a histopathological examination of the thyroid gland was performed in order to detect cytotoxic effects. There was no evidence of inflammation, apoptosis, or necrosis, which are thought to be associated with non-specific increased % tail DNA. Thus, it was concluded that the increase in EMS-induced % tail DNA was due to the genotoxic effects of EMS on the thyroid gland. Based on the above results, it was suggested that EMS could be used as a positive control compound for the thyroid gland in rats, at the same dose exposed to the liver in the *in vivo* comet assay in rats.

EMS is a well-known genotoxic compound that induces DNA damage by a direct mechanism of DNA alkylation (Elmar *et al*., 2009). EMS has been reported to induce tumors in the liver, kidneys, lungs, brain, mammary glands, and uterus in rats (Elmar *et al*., 2009), and has also been reported to cause DNA damage in the stomach, urinary bladder, and bone marrow, where no tumors have been reported (Kitamoto *et al.*, 2015; Kraynak *et al.*, 2015; Uno *et al.*, 2015a, 2015b; Wada *et al*., 2012). Discrepancies in which DNA damage is detected in non-carcinogenic target organs are seen in rats and mice with methyl methanesulfonate, an alkylating agent similar to EMS, *N*-methyl-*N*-nitrosourea, the aromatic amine 2-acethylaminofluorene, and many genotoxic compounds (Leite *et al*., 2007; Miyamae *et al.*, 1998; Sasaki *et al*., 2000; Wada *et al*., 2012). DNA damage may induce chromosomal damage, which is also associated with cancer, or may be repaired and leave no effect, or may be lethal to the cell (OECD, 2016). This may be the reason why the organs in which DNA damage has been observed are not necessarily limited to carcinogenic target organs. Thus, EMS is speculated to induce DNA damage in the thyroid gland, although the thyroid gland of rats exposed to EMS is not a carcinogenic target organ. Therefore, EMS

is expected to be used as a positive control compound in the *in vivo* comet assay of the thyroid gland in rats.

In conclusion, it is considered that EMS is a very useful positive control compound for multi-organ comet assays in rats, targeting the thyroid gland, liver, stomach, urinary bladder, and bone marrow.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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