Comet Assay, a New *in vivo* Mutagenicity Test – Regulatory Significance and Scientific Development

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Ryoko Matsuyama
Keiko Ogata
Sachiko Kitamoto
Mika Oota

A new *in vivo* mutagenicity test, *in vivo* comet assay, has gained particular world wide attention. The comet assay is a promising technique for evaluating *in vivo* DNA damage to multiple organs with high sensitivity. However, there is no validated testing guideline based on the optimized experimental techniques. Recently, to establish a standardized testing method, an international validation study for *in vivo* comet assay has begun with a view to submitting a new OECD test guideline. In this review, we describe the regulatory trends toward this assay for the evaluation of chemical mutagenicity and our investigation of this testing method.

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Introduction

As the demand for development of chemical products with a variety of functions and uses increases, the number, types and quantity of chemical substances used are constantly increasing. Some of these chemical substances adversely affect our health. In order to prevent such chemicals from damaging our health, it is important to conduct accurate safety evaluations and appropriate management of each chemical.

The most serious adverse effects of chemical substances on human health are carcinogenesis and genetic diseases in future generations. Mutagenicity is the potential of a chemical to cause irreversible change on DNA, which is the genetic material of living organisms. If damaged DNA is not restored to its original state, whether it is by a direct or indirect mechanism, it causes mutation of genes or chromosomal aberration. This is one of the triggers for cell carcinogenesis. Therefore, any chemical that has mutagenic properties has a high probability of being a carcinogen. Furthermore, chemicals that cause gene mutation or chromosomal aberration may also demonstrate similar actions in reproductive cells, and may induce genetic diseases in future generations. Animal tests for investigating the potential of carcinogenicity and impacts on future generations are quite expensive and time consuming, and it is extremely difficult to conduct such tests on all newly developed chemical products. For this reason, when handling a new chemical product whose carcinogenicity or potential to cause genetic damage is unknown, mutagenicity is one of the toxicities that must be evaluated beforehand. For new chemical substances, conducting such evaluations before registration is mandatory. The mutagenicity test, a method for detecting the mutagenicity of a chemical substance, is conducted to predict the risk to humans of carcinogenesis and genetic diseases in future generations.

In order to detect genetic damage caused by a variety of mechanisms, several *in vitro* or *in vivo* mutagenicity tests have been developed. These tests can be classified into three categories depending on the indicator used: (1) Methods for detecting gene mutation, (2) Methods for detecting chromosomal aberration and (3) Methods for detecting DNA damage which occurs in the early stage of mutation. Bacteria, cultured mammalian cells and laboratory animals are used for these tests (**Table 1**). It has been revealed that by combining some of the above tests, most mutagens can be detected.

As described above, in order to ensure safety of chemical products (including general chemicals, pharmaceuticals, agricultural chemicals and insecticides for household use), the regulatory authorities in each country require chemical companies to submit the

Table 1 List of mutagenicity tests

Materials	Categories of Mutagenicity Tests		
	Gene Mutation	Chromosomal Aberration	DNA Damage & Repair
Bacteria	•Ames Test	*****	•Rec-Assay
Mammalian Cells	•HGPRT Gene Mutation Test •Mouse Lymphoma Assay	•Chromosomal Aberration Test •Sister Chromatid Exchange Assay	•Unscheduled DNA Synthesis Assay
Animals	•Spot Test •Gene Mutation Assay in Transgenic Mice	•Micronucleus Test •Chromosomal Aberration Test •Sister Chromatic Exchange Assay	•Unscheduled DNA Synthesis Assay •Comet Assay

results of mutagenicity tests for each chemical substance before registration. In recent years, the *in vivo* comet assay, also referred to as the single cell gel electrophoresis assay (SCG), has gained attention as a new *in vivo* mutagenicity test. The *in vivo* comet assay is one technique to detect DNA damage, through which the mutagenicity of a chemical can be evaluated using isolated cells from various types of mammalian organs. Compared to other *in vivo* mutagenicity tests, the method of *in vivo* comet assay is simple. In other existing *in vivo* mutagenicity tests, while the types of organ that can be used for evaluation are limited, many different organs can be used in the *in vivo* comet assay, and it is expected that it can be detected DNA damage in target organs with high sensitivity. It is considered that this technique is a promising new *in vivo* testing method.

In this paper, the current status and issues of the *in vivo* comet assay as a new mutagenicity test are reviewed, and investigations into the *in vivo* comet assay in our laboratory are presented here.

Evaluation of Mutagenicity for Chemical Substances and *in vivo* Comet Assay

Mutagenicity, the ability of a chemical substance to damage genetic materials, is closely related to carcinogenesis and genetic diseases. Evaluation of mutagenicity is extremely important when developing, registering and using new chemical substances. In particular, chemicals that demonstrate mutagenicity in animals may irreversibly alter healthy human DNA and/or chromosomes. For this reason, in the REACH (Registration, Evaluation, Authorization and restriction of CHemicals) chemical substance regulations in Europe, chemicals showing such mutagenicity in animals are classified as Substances of Very High Concern that may seriously damage human health. Handling of these chemicals is strictly controlled in other countries also.

Guideline tests for the evaluation of mutagenicity are as follows: a reverse mutation test using bacteria (Ames test), a gene mutation test using cultured mammalian cells (HGPRT gene mutation test, Mouse lymphoma assay), an *in vitro* chromosomal aberration test using cultured mammalian cells, and a micronucleus test using mice or rats. Because *in vitro* tests (tests using bacteria and cultured mammalian cells) are easy to conduct and have high sensitivity for detection, they are considered essential in the evaluation of the mutagenic potential of compounds. When considering the relevance to humans, *in vivo* tests using animals are given priority.

In the mutagenicity evaluation of a chemical substance, if all mutagenicity test results turn out to be negative, it can be determined that the chemical does not exhibit mutagenicity. However, when a positive result is obtained from a highly sensitive *in vitro* test, even if a negative result is obtained from a first *in vivo* test, which is an essential test (the micronucleus test is usually conducted), it is considered that this is not sufficient evidence to determine that the chemical exhibits no mutagenicity that could cause a serious problem to humans. In this case, additional evaluation must be conducted using a second *in vivo* test.

Conventionally, an unscheduled DNA synthesis test (UDS test) using rodents or a gene mutation test using transgenic animals have been recommended for the second *in vivo* test. The special characteristics of these tests are evaluated in the Guidance on a Strategy for Testing of Chemicals for Mutagenicity [1), 2)] released by the UK COM (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment), one of the European regulatory authorities. These evaluations are described below:

< UDS Test (guideline test) >

- Long history of use and acceptability by regulatory authorities.
- Does not detect mutagenicity resulting from misrepair and non-repair.
- Limited use in tissues other than liver.

< Gene Mutation Test Using Transgenic Animals (no test guidelines) >

- Can be applied to all tissues provided that sufficient DNA can be extracted.
- In general, less sensitive than methods measuring DNA adducts.
- Need for further work to optimize protocols for specific tissues.

Although the UDS test has been most commonly used as the second *in vivo* test, some regulatory authorities have questioned the appropriateness of the UDS test because it is extremely difficult to use organs other than the liver and the number of compounds that show positive is extremely small due to its low sensitivity.

The *in vivo* comet assay is now rapidly gaining attention as an alternative *in vivo* test to the UDS test. The reason is that it is superior to the UDS test in that mutagenicity can be evaluated using various organs, and it is expected to have relatively high sensitivity.

One example is a recently proposed draft guidance of pharmaceutical genotoxicity testing. In April 2008, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) released the Draft Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use S2 (R1) [3]. The purpose of the revision is to optimize the standard genetic toxicology battery. Options 1 and 2 are presented as the standard battery in this proposed draft guidance, and these two options are considered equally suitable. In Option 2, the comet assay was recommended as the second *in vivo* test (**Table 2**).

Table 2 Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2(R1) (Apr.2008 ICH)

Option 1
i. Ames Test
ii. *in vitro* Chromosome Aberration Test or *in vitro* Micronucleus Test or *in vitro* Mouse Lymphoma tk Gene Mutation Assay
iii. *in vivo* Micronuclei or *in vivo* Chromosomal Aberrations Test
Option 2
i. Ames Test
ii. *in vivo* Micronucleus Test and a second *in vivo* assay (Comet assay, Alkaline elution assay, transgenic mouse mutation assays, DNA covalent binding assays, or UDS assay)

* These two options for the standard battery are considered equally suitable.

In addition, a statement which recommends the *in vivo* comet assay as an *in vivo* mutagenicity test has recently been inserted into the guidance stipulated under the EU/REACH regulations (for general chemical substances, implemented in 2007).

In recent years, the "3Rs" (Replacement, Reduce and Refinement) are becoming increasingly important from an animal welfare point of view. It is recommended that the number of animals used in experiments should be reduced as far as possible without affecting the scientific value of the test or the evaluation of risks to humans. The *in vivo* comet assay is relatively easy to combine with other *in vivo* mutagenicity or integrate with general toxicity tests because it requires the minimum amount of tissue sample. In addition, because any animal species can be applied for the *in vivo* comet assay, it does not require special genetically-modified animals as in the gene mutation test using transgenic animals. Therefore, the *in vivo* comet assay is expected to be a promising second *in vivo* test from the 3R principle of animal experiments.

It can be predicted that, in the future, the *in vivo* comet assay will be recommended throughout the world to evaluate mutagenicity of chemical products.

What is Comet Assay?

The comet assay is based on the method developed by Ostling and Johnason in 1984, in which the DNA of individual cells embedded in a gel is subjected to electrophoresis.[4] Subsequently in 1988, Singh *et al.* developed a testing method using an alkaline solution (pH 13)[5], and in 1990 Olive *et al.* developed a modified method of the alkaline comet assay.[6] The comet assay can be classified into two categories: a neutral comet assay and an alkaline comet assay. In the neutral comet assay, electrophoresis is conducted under neutral conditions at pH 7–8, and mainly detects double-stranded DNA cleavage sites and cross-linking sites. In the alkaline comet assay, by conducting electrophoresis under strong alkaline conditions at pH 13 or higher, it is possible to detect a variety of DNA damage, including the single and double-stranded DNA cleavage sites, alkali-labile sites, excision-repair sites and cross-linking sites. At present, the term "comet assay" means the latter alkaline comet assay.

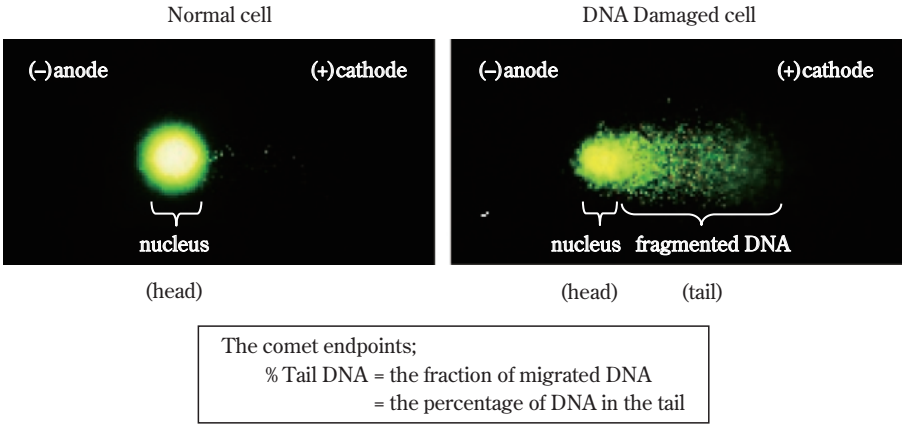


Fig. 1 Single-cell gel electrophoresis assay (Comet assay)

In the comet assay, DNA damage is detected using the following principle: Isolated cells are embedded in a gel and electrophoresed, thus causing the negatively charged DNA to migrate towards the anode. In this electrophoresis, the larger the DNA molecular weight, the less it is for the DNA to migrate. Conversely, the migration is larger for fragmented DNA due to its smaller molecular weight. The comet assay uses this electrophoresis theory to detect DNA damage. When the DNA is not damaged, the molecular weight of the nucleus DNA is very large. Therefore, it maintains its original spherical shape. However, when the DNA is damaged, the DNA fragments caused by the damage will be elecrophoresed, forming a comet-like image (**Figure 1**).

The testing procedures for the *in vivo* comet assay are as follows:

1. The test substance is administered to an animal. The target organs are collected from the animal after administering the compound for a certain period of time, and target cells are then isolated from each organ.
2. The isolated cell is embedded in an agarose gel and then spread on a slide glass.
3. The cell membrane is dissolved, and electrophoresis is then conducted under strongly alkaline conditions.
4. The DNA is then subjected to fluorescent staining, and the fluorescent intensity of the comet image is then measured using a fluorescence microscope and image analyzer (**Figure 2**).

The DNA damage is evaluated as follows: The comet image is first divided into two areas; the head area and

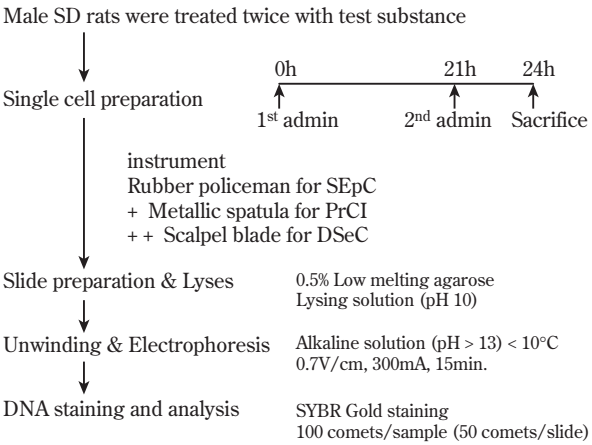


Fig. 2 Alkaline Comet assay

the tail area, which is caused by the electrophoresis. Next, based on the fluorescent intensity of each area, the % tail DNA (tail intensity: the ratio of the brightness (*i.e.* the DNA content) of the tail area to that of the entire comet) is then calculated. The comet assay is evaluated as to whether this value is significantly higher than the control group.

The advantages of the *in vivo* comet assay are as follows:

– A variety of organs can be used.
– An experimental procedure is relatively simple.
– It can be expected to detect DNA damage in the target organs with high sensitivity.
– It can contribute to the reduction of laboratory animals used.

On the other hand, testing guidelines for the *in vivo* comet assay have not yet been developed, and no testing method has been standardized. The testing procedures together with the data analysis vary depending on researchers, leading to inconsistent results. In

order for the comet assay to be regarded as a reliable *in vivo* test, it is essential to standardize the testing method. To avoid conducting unnecessary animal experiments from the 3R perspective, it is necessary to establish a unified, reliable testing method and develop testing guidelines.

JaCVAM International Validation Study

In response to a growing need for standardization of the *in vivo* comet assay, standard methods for the assay were discussed at the International Workshop on Genotoxicity Test Procedures (IWGTP) and the International Comet Assay Workshop (ICAW). The details of the discussions held at each workshop, including the variations and validity of the testing methods, were later reported by Tice *et al.* (2007)[7] and Hartmann *et al.* (2003)[8]. Thus, the establishment of a standard method for the *in vivo* comet assay started.

Under these circumstances, international validation study of the *in vivo* comet assay was initiated in August 2006 by the Japanese Center for Validation of Alternative Methods (JaCVAM). This validation study is currently being implemented with the support of the organizations listed below:

- The Japanese Environmental Mutagen Society/ Mammalian Mutagenicity Study Group (MMS/ JEMS)
- The European Center for the Validation of Alternative Methods (ECVAM)
- The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
- The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

The purpose of the international validation study is to standardize the protocol and validate the availability of the *in vivo* comet assay for evaluation of mutagenicity. The ultimate goal is to propose OECD testing guidelines for the *in vivo* comet assay. By the year 2008, Phases 1, 2 and 3 of the pre-validation test had been conducted by the lead laboratories, and testing methods and acceptable criteria *etc.* had been investigated. Then, to conduct Phase 4 of the main validation test, the participation of the facilities in this phase was called upon.

As previously described, taking into account the EU/REACH regulations and trends in regulations for agricultural chemicals and insecticides for household use that may be implemented in the next few years, we recognized the importance of the *in vivo* comet assay and considered that we should proactively make use of the international validation study. We have decided to participate in Phase 4 for the following reasons: Through the international validation study, it can be confirmed whether the *in vivo* comet assay is a useful test system compared to the UDS test and the transgenic test; through our participation in the international validation study, we will have easier access to the latest technology and protocols at all times; and by establishing good connections with external researchers, timely and accurate information can be obtained. The requirements for the participation in Phase 4 are listed below:

1) The study will be conducted in facilities that are GLP compliant.
2) Using a specified image analyzer system to collect DNA migration data.
3) Sufficient experience to submit the historical data on at least five chemicals.

* With respect to 3), facilities which have only minimal experience of the *in vivo* comet assay must conduct the pre-validation test shown below and submit the data to the Validation Management Team (VMT) under the JaCVAM. After a data audit by the VMT, the facility in question will be qualified to participate in the international validation study.

Pre-validations 1 and 2: Positive control, ethyl methanesulfonate (EMS), repeat tests

Pre-validations 3 and 4: Two coded compounds

Since we did not satisfy the criteria 3), we had to conduct the pre-validation test. Upon conducting the pre-validation test, we established the testing method for the comet assay in a glandular stomach. The details of these examinations will be described in the next section. Based on the result of these examinations, we conducted pre-validations 1 and 2 (the *in vivo* comet assay of a positive control substance). Our results fulfilled the criteria for both the positive and negative control substances, proving that there is no problem to conduct the assay at our laboratory (**Figures 3** and **4**). We have submitted the data from pre-validations 3 and 4 (the *in vivo* comet assay of coded compounds) to the VMT, thereby qualifying for participation in the international validation study.

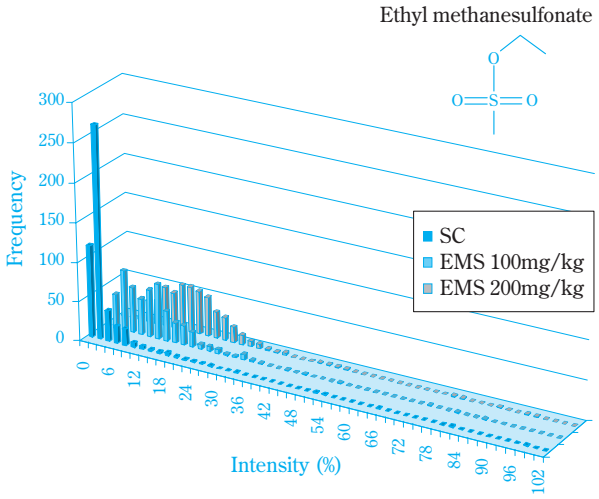


Fig. 3 DNA damage in liver induced by EMS

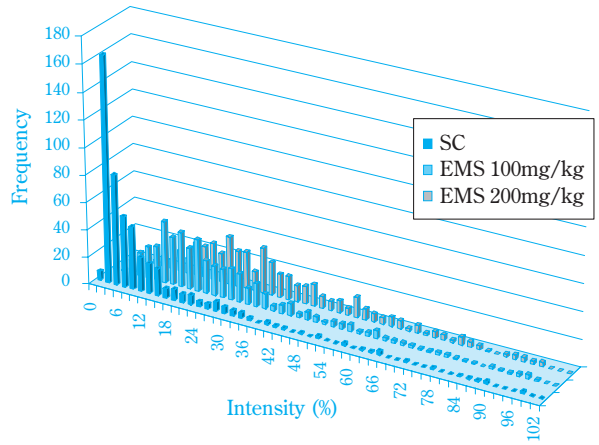


Fig. 4 DNA damage in glandular stomach induced by EMS

Once the participating facilities, including our company, had been selected, Phase 4 of the validation test (30–50 compounds to be evaluated by the 13 participating facilities) began in May 2009. Phase 4 of the validation test is currently in progress with a completion goal of the end of 2010.

Examinations of *in vivo* comet assay using glandular stomach

The target organs/tissues used for the *in vivo* comet assay in the validation study are the liver and glandular stomach. The liver plays a major role in metabolism and is an organ typically used when investigating the systemic effects of both the compound and its metabolites. Since the stomach first comes into direct contact with the compound when orally administered, it is considered that the compound is present at a relatively high concentration. For this reason, the stomach is selected as a representative organ used for investigations into the direct and local effects of the compound. The liver is composed of several homogeneous cells, and the area chosen for cell collection does not particularly affect the result of the test. On the other hand, methods for isolating cells from the glandular stomach–in which differentiated cells have a layered structure–have never been previously investigated in detail. Therefore, we investigated methods for appropriately collecting cell layers for the evaluation of the *in vivo* comet assay.

The tissue structure of the glandular stomach is explained as follows. The glandular stomach consists of the cardiac glands adjacent to the esophagus and the forestomach, the fundal gland, which is the main component of the gastric mucosa, and the pyloric gland, which is connected to the duodenum (**Figure 5**).

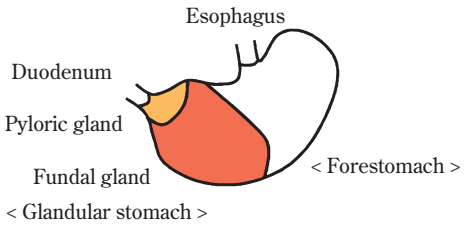
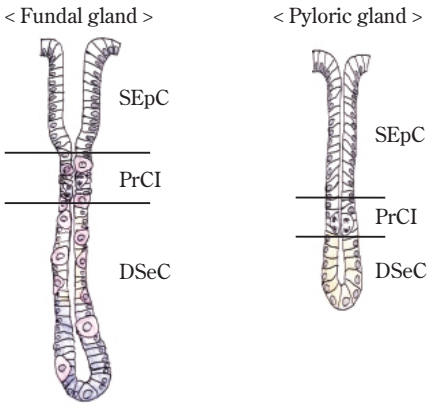


Fig. 5 Compartmentalization of rat's gastric mucosa

The mucous layer of the glandular stomach can be roughly divided into three zones: the surface epithelial cell zone (SEpC), the proliferating cell zone in the isthmus (PrCI) and the glands. After proliferation, the undifferentiated cells in the PrCI, which are located slightly deeper from the luminal surface, migrate to the SEpC or to the deeper glands, which is the differentiated secretory cell zone (DSeC), while at the same time maturing (**Figure 6**). While the lifetime of the gastric mucosal cells (parietal cells and chief cells of DSeC) is about 200 days, that of the cells in the SEpC is about 3 days. Because the glandular stomach is a tissue that secretes mucus, and the cells in the SEpC are replaced with new cells together with the mucus within a few days, the SEpC contain many cells in which cell death (apoptosis) has occurred. In the comet assay, the DNA damage caused by apoptosis and that caused by mutagenicity cannot be distinguished. Because the lifetime of the surface epithelial

cells is short, it is considered that the possibility of carcinogenesis in these cells is low, and these cells are therefore not suitable for evaluation of mutagenicity. On the other hand, the proliferating cells present in the lower layer of the surface epithelial cells are undifferentiated and undergo vigorous cell division, and it is highly possible that these cells will turn into cancer cells if DNA damage has occurred. Therefore, it is considered that these undifferentiated cells in the PrCI should be used as the target cells for the *in vivo* comet assay.



SEpC : Surface epithelial cell zone, containing columnar epithelial cells
PrCI : Proliferating cell zone in isthmus
DSeC : Differentiated secretory cell zone, containing differentiated secretory cells

Fig. 6 Target cells of glandular stomach

According to the validation protocol presented by VMT, the method for isolating undifferentiated cells from the glandular stomach was not described sufficiently and depended greatly upon the way each scientist handled the tissue. Also, under this protocol, no confirmatory testing was conducted to check whether only undifferentiated cells had been isolated. Therefore, we have conducted the following investigations into the *in vivo* comet assay using a glandular stomach.

< Investigation into Methods of Cell Collection for Glandular Stomach >

Methods for collecting the target cells from the glandular stomach were investigated using tools of various materials: a silicone rubber scraper (cell scraper for dish plates manufactured by IWAKI), a metallic spatula and a scalpel blade. The strength and the number of scraping repetitions for each cell scraping tool were altered, and the collected mucosal cell layers were observed. To confirm the exfoliation of the mucosal layer, pathological samples were prepared from the tissues removed by scraping with the tools, and histopathological analysis was then conducted on each sample. As a result, it was found that by using a silicone rubber scraper alone, while SEpC was removed, few PrCI were removed even if the strength and the number of scraping repetitions were increased. Therefore, almost all cells in the SEpC were removed using a silicone rubber scraper. The same area was then scraped using a metallic spatula. With this method, the target cells in the PrCI were successfully isolated. Furthermore, it was found that by using a scalpel blade for scraping after using both a silicone rubber scraper and a metallic spatula, DSeC in the lower layer of the proliferating cell zone could be collected (**Figure 7**).

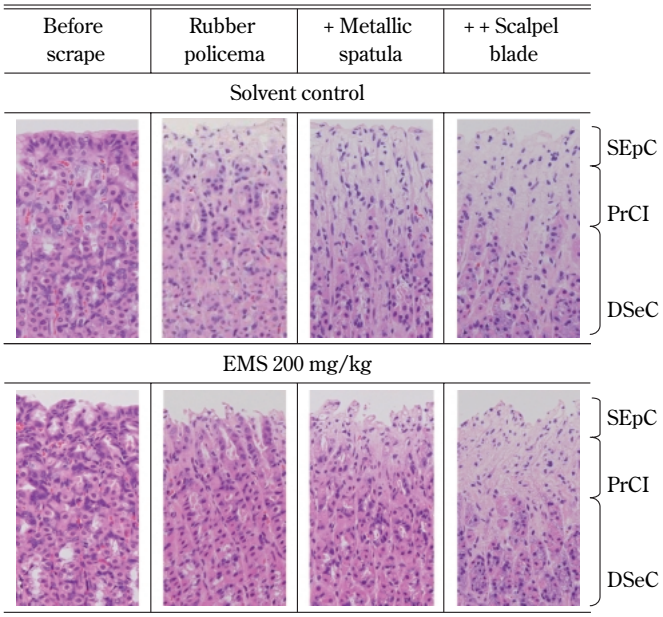


Fig. 7 Histopathological analysis –Fundal gland

< *In vivo* Comet Assay Using Cells from Each Layer of the Glandular Stomach >

Once the cells from the SEpC, PrCI and DSeC had been successfully collected using the appropriate cell collection tools, the effects of the target cells in the PrCI and other non-target cells on the comet assay were investigated. The EMS which is commonly used as a positive control substance was used as a test compound. Cell suspensions were obtained from SEpC, PrCI and DSeC of the fundal gland of the same animal. The comet assay was then conducted so as to

compare the extent of DNA damage of each cell layer. As a result, although the DNA of the negative control group was not damaged, the %Tail DNA value of the SEpC was higher than that of the PrCI and DSeC. This was because the SEpC contains many cells in which cell death (apoptosis) has occurred. Even though the DNA of the cells in the SEpC had not been damaged by the compound, DNA fragmentation had been caused by apoptosis. With regard to the reaction to the positive control substance when using any of the cells, a significant increase relative to the negative control group was observed, as well as a dose dependency. The ratio of the positive and negative control groups did not differ significantly in any of the cells (**Figure 8**).

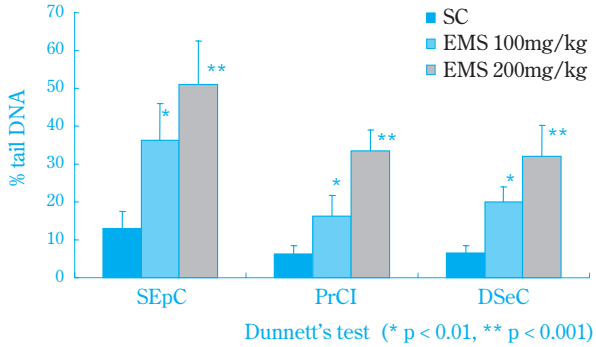


Fig. 8 DNA damage of the layers in fundal glands induced by EMS

< *In vivo* Comet Assay Using the Fundal Gland and Pyloric Gland >

The glandular stomach consists of the cardiac gland, the fundal gland and the pyloric gland. The cardiac gland hardly exists in rats. In the *in vivo* comet assay, the fundal gland and the pyloric gland are not distinguished, and cells are collected from both glands. Because it was previously reported that the sensitivity of the fundal gland toward carcinogenic substances is higher than that of the pyloric gland [9], cells in the PrCI were collected from the fundal gland and the pyloric gland, and the effect on the *in vivo* comet assay of these cells was investigated using a positive control substance (EMS). For the pyloric gland, after scraping the cells in the SEpC off using a silicone rubber scraper, the cells in the PrCI were collected using a metallic spatula. To confirm the exfoliation of the mucosal layer of the pyloric gland, a pathological sample was prepared, and histopathological analysis was conducted (**Figure 9**). As a result, it was found that the %Tail DNA values of the positive and negative control groups were almost the same in the fundal gland and the pyloric gland, thus showing no difference in the extent of DNA damage of these glands (**Figure 10**).

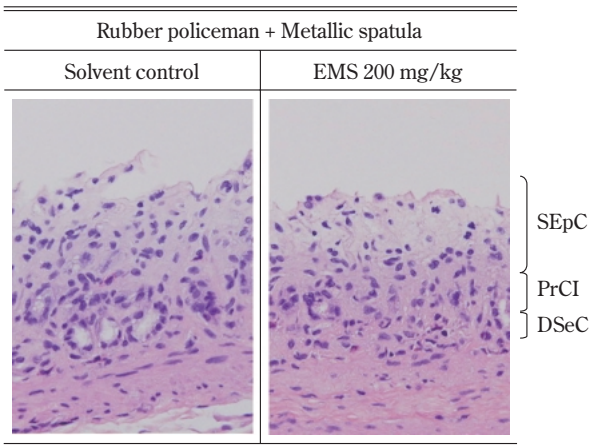


Fig. 9 Histopathological analysis –Pyloric gland

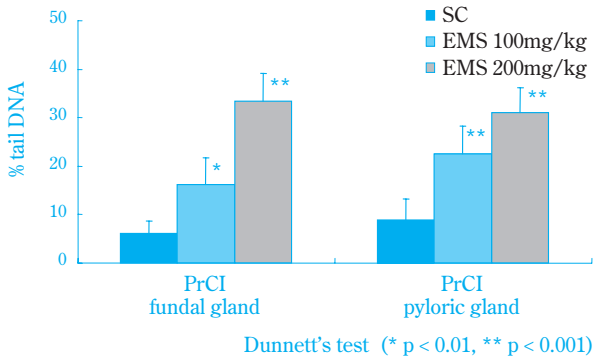


Fig. 10 DNA damage in the fundal gland and the pyloric gland induced by EMS

< *In vivo* Comet Assay Using 2,6-DAT >

From the above results obtained, it has become clear that the variations in the mucosal cell layers of the glandular stomach (i.e., the SEpC and PrCI) affect the %Tail DNA value. Because the SEpC is located on the luminal surface, and the SEpC contains many apoptotic cells, the %Tail DNA values of the SEpC the negative and positive control groups were higher than that of the PrCI. For the purpose of comparison, the comet assay was conducted on the SEpC and PrCI using compounds other than EMS that showed clear positive response. 2,6-diaminotoluene (2,6-DAT, CAS No. 823-40-5) was used as the test compound. According to previous findings for 2,6-DAT, although most of the *in vitro* mutagenicity tests turned out to be posi-

tive, most of the *in vivo* mutagenicity tests showed negative. Carcinogenicity in rodents was not observed. Sekihashi *et al.* reported (2002)[10] that in the *in vivo* comet assay, no significant increase was observed in any organs (stomach, colon, liver, kidney, lung, bladder, brain or bone marrow) of rats or mice. We conducted the *in vivo* comet assay using the cells in the SEpC and PrCI. As a result, while no significant increase were observed in the PrCI in either the 2,6-DAT administered group or the negative control group, a significant increase was observed in the SEpC of the 2,6-DAT administered group (250mg/kg) compared to the negative control group (Dunnett's test, one-sided, $p < 0.05$) (**Figure 11**). It is considered that because the SEpC contain many apoptotic cells, this fluctuation in the apoptotic cell content may have affected the result, and may have accidentally caused the significant difference. Alternatively, this result may suggest that DNA damage in the SEpC located on the luminal surface occurred due to the effects of the 2,6-DAT. However, even though DNA damage has occurred in the SEpC, taking into account that the lifetime of these cells is about 3 days, meaning that the cell turnover is quick, it is not likely that such DNA damage will cause cancer in the future. Undifferentiated cells in the PrCI are highly likely to transform into cancer cells due to DNA damage. The PrCI should be employed in mutagenicity tests using the stomach for the purpose of carcinogenicity screening. In fact, from the fact that 2,6-DAT does not exhibit carcinogenicity and from the results of the SEpC in the comet assay, it is considered that the results of the PrCI in the comet assay have a stronger correlation with carcinogenicity than the results of the SEpC in the comet assay. Although the 2,6-DAT case is one example, the test results suggested that if SEpC which does not contain target cells is included, false results may be obtained. Therefore, it is considered that using appropriate cells contributes to improving the reproducibility and the reliability of the *in vivo* comet assay. This would also enhance the significance of carcinogenicity screening tests. Our successful establishment of a method for collecting cells in the PrCI by removing the SEpC is significant to verify the sensitivity and the reliability of *in vivo* comet assays as well.

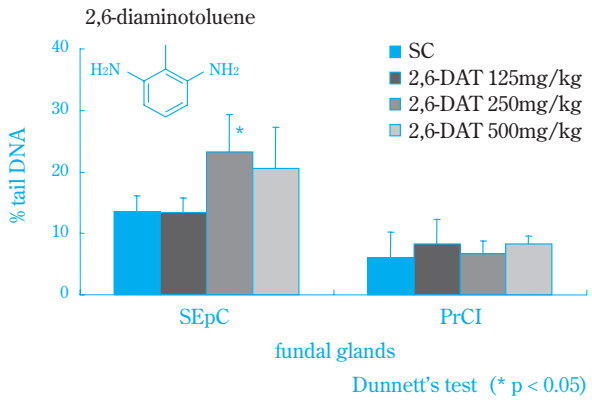


Fig. 11 DNA damage of the layers in fundal gland induced by 2,6-DAT

Conclusion

Some chemical substances directly or indirectly affect the DNA structure, thus causing DNA damage, errors in DNA repair, gene mutations and chromosomal aberrations. Gene mutations and chromosomal aberrations can act as triggers for various diseases such as cancer and teratogenesis. For evaluating the mutagenicity of chemical substances, the *in vivo* comet assay is becoming noticed as a test that can be an alternative to the conventional unscheduled DNA synthesis tests (UDS tests) that have been most commonly used as the second *in vivo* test until now. In Europe, some regulatory authorities already require *in vivo* comet data for the registration of compounds. In Japan, the regulatory authorities have started to pay attention to the availability of the *in vivo* comet assay. In order to establish the *in vivo* comet assay as the appropriate second *in vivo* assay, it is essential to optimize and standardize the testing methods. In the future, once the reliability of the *in vivo* comet assay has been confirmed and OECD testing guidelines are developed, it is expected that the regulatory authorities strongly request the data submission.

We havecaught changes in trends of regulations in a timely manner and proactively took part in the international *in vivo* comet assay validation study to develop the OECD guideline. At our laboratory, we conducted pre-validation tests and accumulated background data. Furthermore, in order to optimize the testing method, we investigated the method for collecting glandular stomach cells, and established a collecting method for the PrCI, which is considered to be the most appropriate region for testing. We continue to inspect the availability of the *in vivo* comet assay and to monitor future global trends in regulations, while at the same time participating in the Phase 4 of the international validation test that began in 2009.

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PROFILE

Ryoko Matsuyama

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Researcher

Sachiko Kitamoto

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Senior Research Associate

Keiko Ogata

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Researcher

Mika Oota

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Senior Research Associate