NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

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National food safety standard - In vivo mammalian alkaline comet assay

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1 Scope

This Standard specifies the basic test methods and technical requirements for in vivo mammalian alkaline comet assay.

This Standard is applicable to the evaluation of the DNA damage effect of the test substance on mammalian tissue cells.

2.1 Comet

Comet refers to the tailing phenomenon of damaged DNA fragments under the action of an electric field, which looks like a "comet" in the microscopic image. The comet head is nuclear DNA; the comet tail is composed of damaged DNA fragments that migrate out of the nucleus in an electric field.

2.2 Scorable Cell

Scorable cell refers to a cell with a clear outline of comet head and tail and is not disturbed by neighboring cells.

2.3 "Hedgehog" Shaped Cell "Hedgehog" shaped cell refers to a cell that consists of a small or fuzzy head and a large and diffused tail in the microscopic image.

2.4 DNA Percentage of Tail

DNA percentage of tail refers to the ratio of DNA content of the comet tail to the total DNA

content (the sum of the head and tail). It reflects the relative degree of DNA damage, which is expressed as a percentage.

2.5 Key Variables

Key variables refer to test parameters, whose minor changes may have a relatively significant influence on the test results. Key variables include sampling time, lysis conditions and electrophoresis time, etc. The key variables may be tissue specific.

4.2.4 Electrophoresis buffer. weigh-take EDTA disodium salt and sodium hydroxide (analytically pure); use pure water to prepare a solution (final concentrations are. 0.001 mol/L EDTA disodium salt and 0.3 mol/L sodium hydroxide). Prepare it right before use.

Adjust pH to make pH >= 13.Before use, keep it refrigerated at below 10 °C for storage.

4.2.5 Neutralization buffer. weigh-take tris (hydroxymethyl) methyl aminomethane (analytically pure); use pure water to prepare a solution; the final concentration is 0.4 mol/L. Adjust pH to 7.5.Before use, keep it refrigerated at below 10 °C for storage.

4.2.6 Shearing buffer. weigh-take EDTA disodium salt (analytically pure); use Hank's balanced salt solution (HBSS) (pH 6.7 ~ 7.8, excluding Ca2+, Mg2+ and phenol red) to dissolve it and prepare a solution; the final concentration is. 0.02 mol/L. Adjust pH to 7.5.Right before use, add dimethyl sulfoxide; the final concentration is 10% (V/V).

Before use, keep it refrigerated at below 10 °C for storage.

4.2.7 Staining solution. DNA fluorescent dyes (for example, SYBR Gold, SYBR Green I, Gelred, propidium iodide or ethidium bromide); in accordance with the product requirements, prepare and use it.

5 Test Methods

5.1 Test Substance

5.1.1 Method of preparation. the test substance shall be dissolved or suspended in a suitable solvent. The optimal solvent is water. For test substances that are insoluble in water, vegetable oils (such as. olive oil and corn oil, etc.) may be used. For test substances that are insoluble in water or oil, carboxymethyl cellulose and starch may also be used to prepare suspensions or pastes. The test substance shall be prepared right before use, unless there is data suggesting that its solution or suspension is stable in storage.

5.1.2 Administration route. the test substance shall be administrated by gavage.

Generally speaking, the gavage volume does not exceed 10 mL/kg body weight for rats and 20 mL/kg body weight for mice. If it is an aqueous solution, the maximum gavage volume may reach 20 mL/kg body weight; if it is an oily liquid, the gavage volume shall not exceed 4 mL/kg body weight; the gavage volume shall be consistent in each group.

5.2 Positive Control The positive control substance shall be able to induce DNA strand breaks in the target tissue of the test substance. Ethyl methane sulfonate (EMS) may be selected as the positive control substance. The mode, in which, the positive control substance is handled is not necessarily the same as that of the test substance. The positive control substances and their corresponding target tissues (for rodents) are shown in Appendix A.

group shall follow the following sequence.

a) 10 g/kg body weight;

b) 100 times of possible intake by human beings;

c) Maximum gavage volume at one time.

In addition, there is also a negative (solvent) control group. The positive control substance may be administrated by oral gavage of 200 mg/kg body weight of ethyl methane sulfonate.

6 Test Procedures

6.1 Animal Observation During the test, observe and record animal health at least once a day (preferably at the same time). Meanwhile, the peak period of expected effects after the test substance is administrated shall be taken into consideration.

6.2 Sampling Time

6.2.1 The sampling time is a key variable, which shall be after the DNA strand is induced to break, and before the break is removed and repaired, or before cell death.

The duration of some damages that cause DNA strand breaks detected by the alkaline comet assay is probably extremely short. If such transient DNA damage is suspected, then, measures shall be taken to reduce the leak detection of DNA damage and ensure that tissue sampling is carried out as soon as possible, which is probably earlier than the sampling time provided below.

6.2.2 The best sampling time depends on the test substance itself or the mode, in which, the test substance is administrated. When conditions permit, the sampling time shall be determined by the toxicokinetic data [for example, reaching the peak plasma or tissue concentration (Cmax) and time (Tmax), or the steady state of multiple administrations of the test substance]. In the absence of the toxicokinetic data, the method of administrating the test substance (including the positive control substance)

to the animals twice (with an interval of 21 h) is often adopted. 3 h after the last administration of the test substance, conduct one-time sampling. When the alkaline comet assay is integrated with the repeated feeding test (for example, the 28-day oral toxicity test), the same applies to the one-time sampling 3 h after the last administration of the test substance.

The test may also adopt the following two sampling modes.

a) The test substance is administrated to the animals once; 2 h ~ 6 h and 16 h ~

26 h after the administration of the test substance, conduct sampling twice.

The preparation of glass slides shall be completed within 1 h after the preparation of the single cell / nucleus suspension.

When preparing the glass slides, the amount of single cell / nucleus suspension added to the low melting-point agarose (usually, 0.5% w/V ~ 1.0% w/V) shall not reduce the concentration of the low melting-point agarose to 0.45% w/V. The optimal cell density (cells are not overlapped, and with a concentration convenient for image analysis) shall be determined in junction with the image analysis system of comet scoring.

6.6 Lysis The lysis conditions are a key variable. In the same test, the lysis conditions of all slides shall be kept as consistent as possible.

Immerse the slides in the pre-cooled cell lysis solution (the lysis solution shall cover the slides); at 2 °C ~ 10 °C, perform lysis in the dark for at least 1 h (or overnight). After lysis, rinse the slides to remove the residual detergent and salt. For rinsing, pure water, neutralization buffer or phosphate buffer may be used, or electrophoresis buffer may also be used.

6.7 Unwinding and Electrophoresis Randomly place the slides on a horizontal electrophoresis tank containing enough electrophoresis buffer. The electrophoresis buffer shall cover the slides (the coverage depth of the electrophoresis buffer shall also be consistent each time). The slides shall be placed for at least 20 min to unwind the DNA, then, set the electrophoresis conditions. The electric potential is usually 0.7 V/cm; the electrophoresis time is not less than 20 min. The electrophoresis time is a key variable, whose dynamic range needs to be optimized. In each test, the voltage shall be kept constant, and the other parameters shall change within a specific narrow range. During the whole electrophoresis process, the depth of the electrophoresis buffer shall be ensured; the current at the beginning and the end of electrophoresis shall be recorded. Unwinding and electrophoresis are performed at 2 C ~ 10 °C in the dark.

The position where the slides are placed in the electrophoresis tank shall be balanced, so as to reduce the influence of edge effects and minimize the difference between batches. In each electrophoresis, samples for the negative control and positive control in different dose groups shall have the same number of slides.

6.8 Neutralization and Dehydration After the electrophoresis is completed, take out the slides. Use the pre-cooled neutralization buffer to rinse them for at least 5 min. Then, naturally dry them in the air or completely dry them at 37 °C. Under the circumstance when slide reading needs to be extended, the slides may be immersed in absolute ethanol, which is pre-frozen to -20 °C to stabilize for 5 min, dried and stored at room temperature, or