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STUDY OUTLINE

Rodent Alkaline Single Cell Gel Electrophoresis (in vivo Comet Assay)

Objective	The Comet assay is a simple method for measuring DNA strand breaks in eukaryotic cells. Cells embedded in agarose on microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks and the size of the resulting fragments.
Animal species, sex	Rat, CRL (WI) BR of Wistar origin, (young adult male rats, less than 8 weeks old at the commencement of the treatment).
Number of animals	The required minimum is 5 scorable animals/groups (test item treatment, negative and positive controls).
Dose levels	A minimum of three (MTD and lower doses), depending on the results of the dose-range finding study.
Controls	Positive and negative (vehicle) controls investigated parallel with the test item doses.
Administration of doses	Administration per oral gavage, two administrations separated by 24 hours, with one sampling time 3 to 6 hours after the last dose administration.
Tissues to be examined	First site of contact (stomach, duodenum) and liver; but the organ selection depends on the main test item target.
Preparation of single cells	Examination of whole cells, mincing briefly the solid organ with a pair of scissors (mincing buffer: HBSS, containing 20mM EDTA and 10% DMSO) preferred, however tissue incubation with digestive enzymes (e.g.: collagenase, trypsin) is feasible in the testing laboratory. Tissue preservation: samples for a potential histopathology-in case of positive effect. Viability (cytotoxicity) assessment by dual dye staining technique using 5,6-carboxyfluorescein diacetate and ethidium bromide.
Processing of slides	Pre-treatment of microscope slides, embedding of cells at 10^4 order (using low and normal melting point agarose).
The Comet assay	*Lysis* (Lysing solution: 2.5M NaCl, 100mM EDTA, 10mM Tris, 12g/L NaOH; 10g/L N-Laurylsarcosyl; 10% DMSO, 1% Triton X-100; 1 hours ≤, at ~4^{o}C); *Unwinding* (Electrophoresis solution: 300mM NaOH, 1mM EDTA; pH>13; 20-45 min at pH>13; at ~4°C); *Electrophoresis* (Electrophoresis solution; 20-40 min, at 25V/300 mA); *Neutralization* (0.4M Tris, pH=7.5; 3 x 5 min.; dehydration: abs. ethanol) *Staining* (2µg/mL Ethidium bromide)
Number of slides prepared	At least 15 slides per treatment per organ for the treatment groups and controls
Number of slides evaluated	50 cells per slide will be randomly scored *i.e.* 150 cells per animal (750 analyzed cells per test item treatments and controls).

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Evaluation

The slides are examined with an appropriate magnification using fluorescent microscope equipped with an appropriate excitation filter. For Image Analysis (analysis of tail length, tail moment, tail intensity) the Komet 6.0 F (Andor Technology) is used.

Draft Report

Approximately 4 months from the arrival of the test item.

Archiving

Study Plan, amendment(s), original final report and all raw data, and one sample of the test item for 5 years

References

Hartmann A., Agurell E., Beevers C., Brendler-Schwaab S., Burlinson B., Clay P., Collins A., Smith A., Speit G., Thybaud V., Tice R.R.: Recommendations for Conducting the *in vivo* Alkaline Comet Assay, Mutagenesis 18:45-51 (2003)

Burlinson B., Tice R.R., Speit G., Agurell E., Brendler-Schwaab S.Y., Collins A.R., Escobar P., Honma M., Kumaravel T.S., Nakajima M., Sasaki Y.F., Thybaud V., Uno Y., Vasquez M., Hartmann A.: Fouth International Workgroup on Genotoxicity Testing: Results of the *in vivo* Comet Assay Workgroup, Mutation Research 627:31-35 (2007)

International Validation of the in vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Version 14.2, November 30, 2009 revised) proposed by EFSA (EFSA Journal 2012; 10(11): 2977

Option 1

Extension of archiving: Study Plan, Amendment(s), original Final report and all raw data for further 10 years; biological samples for further 7 years.