6th International Workshop on Genotoxicity Testing (IWGT)
Foz do Iguaçu, Brazil, October 31st to November 2nd, 2013

Report from the Comet Assay Working Group:

"Critical Issues with the *in vivo* Comet Assay"

Members of the working group:
Chair: Günter Speit
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Peter Kasper
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Yoshifumi Uno
Marie Vasquez

Invitated participants:
Carol Beevers
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Note:

- **This presentation may not always use the exact wording on which IWGT agreed.**
- **The "official" conclusions will be published in a special issue of Mutation Research.**

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Introduction

- The comet assay (single cell gel electophoresis) is a test for the detection of primary DNA damage which is widely used in basic research, biomonitoring and genotoxicity testing.
- IWGT is only concerned with the ***in vivo*** **assay** and its use in **regulatory genotoxicity testing**.
- A draft OECD guideline has been written and is being revised after a first commenting round.

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Previous IWGT activities:

4th IWGT (San Francisco, 2005): In vivo comet assay workgroup

Burlinson et al., Mutat. Res. 627, 31-35 (2007)

Discussion topics:

- Multiple dose levels versus limit dose.
- Cell isolation process.
- Image analysis or manual scoring.
- Historical control data.
- Minimal reporting standards.
- Concurrent measures of cytotoxicity.

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5th IWGT (Basel, 2009): In vivo genotoxicity testing

Rothfuss et al., Mutat. Res. 723, 108-120 (2011)

Discussion topics (related to the comet assay)**:**

- Combination of the MN assay and comet assay into acute studies.
- Integration of comet assays into repeated-dose toxicity (RDT) studies.

Conclusion:

The combination / integration of MNT and comet assay is scientifically justified for both acute and RDT studies.

- More data are needed for compounds with diverse modes-of-action.
- Test compounds with extra-hepatic target tissues.
- The use of frozen cell samples for the comet assay.
- Need to provide historical control data.

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Proposed topics for discussion at the 6th IWGT:

- Domain of applicability:
  Which types of DNA damage are detected / missed?
- Attempts to standardize the comet assay:
  What is necessary? Which information has to be provided?
- Reproducibility of results and assay variability.
- The role of positive and negative controls.
- Measures of cytotoxicity:
  Impact on test results / interpretation of studies?
- Experience with combination and integration.
- Regulatory acceptance of studies.

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Presentations:

- Yoshifumi Uno
  Towards an OECD guideline – critical issues.
- Andrew Collins
  How much standardization and calibration is needed?
- Marie Vasquez
  Cytotoxicity – measures and impact on test results.
- Brian Burlinson
  Intra- and inter-laboratory reproducibility of comet assay results.
- Ulla Plappert
  The comet assay in current test strategies: combination and integration.
- Peter Kasper
  What makes comet assay acceptable to regulating agencies?

Conclusions (I):

The comet assay is mature enough to produce reliable results

- For regulatory purposes should only be performed by laboratories that have demonstrated proficiency.
- The assay will be used in different contexts such as for screening and to follow up positive *in vitro* results.
- The 2008 analysis by Kirkland and Speit* showing increased sensitivity of comet over UDS is supported by recent experience testing drugs.
- There are some things we can do to further develop the assay in future but they should not impede use of the assay.

*) Kirkland & Speit: Mutat. Res. 654 (2008) 114-132.

Conclusions (II): Domain of applicability

- When the lesion is unknown the assay is useful as part of a battery to detect DNA damaging agents.
- Crosslinking may be detected by a decrease in migration but the standard protocol is not designed for this purpose and may need to be altered to reliably detect this class of damage.
- The standard protocol detects many but not all types of DNA damage; bulky adducts are an example of a lesion class which might be missed.
- When the expected lesion can be predicted (e.g. oxidative damage, bulky adducts), it is possible to modify the protocol using enzymes that recognize the damage and therefore expand the domain of applicability.

Conclusions (III): Extension to tissues beyond liver

- There is most confidence of use of the assay in the liver.
- Experienced labs have demonstrated reproducible vehicle control responses reliable detection of positive controls in several other tissues.
- Some tissues are easier to use than others.

Conclusions (IV): Critical protocol parameters

- Treatment schedule and sampling times:
  - Integration of comet into repeat dose studies has been demonstrated to be feasible.
  - Most experience to date however is with use of the assay, alone or including micronucleus, with an acute protocol used as a follow-up to positive *in vitro* result.
- There are certain critical parameters in the protocol which will influence the test result.
  The best way to demonstrate adequate control of these parameters is examination of the contemporaneous and historical positive and vehicle control data.

Critical protocol parameters

- Agarose concentration
- Alkaline incubation
- Voltage (V/cm)
- Electrophoresis time

Azqueta et al., Mutat. Res. 724 (2011) 41-45.

Conclusions (V):
Demonstration of power and sensitivity-laboratory proficiency

- Demonstration of laboratory proficiency requires demonstration of adequate vehicle controls and an adequate dose response to a positive control (e.g. EMS) for each tissue being examined.
  - Laboratories should track response to the positive control and vehicle controls over time.
  - Laboratories should calculate the power of their protocol to reliably detect an two fold increase as statistically significant with an acceptable level of power (e.g. 60, 80 or 90% of the time).

Conclusions (VI): Freezing of cells

- Some labs have successfully demonstrated consistent results with freezing samples for various periods of time prior to analysis and have published those results*.
- Other laboratories have had trouble demonstrating reliable results after freezing samples.
- Generally accepted protocols for freezing are not yet available.
- Freezing often increases variation from sample to sample, especially in treated animals, which may reduce fold increase (i.e. sensitivity).

*) Recio et al.: Environ. Mol. Mutagen. 53 (2012) 101-113.
Jackson et al.: Mutagenesis doi:10.1093/mutage/get049 (2013).

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Conclusions (VII): Tissue toxicity

- Toxicity could be a confounder of comet results.
- It is useful to look at as many measures of toxicity as possible including organ-specific clinical chemistry as well as a variety of histopathological observations to see if compound-specific toxicity might influence the result. Combination of all available observations can contribute to evaluation of the result.
- So far, observations have been compound specific.
- However, we do not yet know if or whether any of these measures of tissue toxicity correlate with false negative or false positive results.
- Tissue toxicity has been observed in association with reduced comet response relative to non toxic doses and may indicate the possibility of a causative effect.
- When toxicity is observed at all concentrations tested, further study at non-toxic doses may be advisable.
- Hedgehogs can be the result of both toxicity and DNA damage and are thus not a reliable index of tissue toxicity.

Acknowledgements

- **The IWGT Comet Assay Workgroup.**
- **All who took part in the discussions.**
- **David Kirkland and Hansjoerg Martus.**