The HET-MN (Hen’s Egg Test for Micronucleus-Induction): Promising pre-validation study

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The HET-MN allows the analysis of micronuclei formation in erythrocytes of developing hen’s eggs to address the systemic availability of compounds as it facilitates the absorption, distribution, and metabolism of compounds followed by their excretion into a bladder equivalent. Here we present the results of >30 compounds, tested blinded in a pre-validation exercise in 3 laboratories. The test set covers true positives and negatives (concordant historical in vitro a. in vivo data) as well as misleading positives (positive in vitro, negative in vivo). Data analysis resulted in a specificity of 97% and a sensitivity of >80%. Studies on xenobiotic metabolism proved the clear intrinsic metabolic capacity of the test system, which omits the need to add rat liver 59 mix. According to our findings, the HET-MN is a promising assay to supplement existing in vitro genotoxicity test batteries to follow up on initial positive results.

Abstract/introduction

- Good overall predictivity of method. High specificity of pre-validation data set of 97%, which is a prerequisite for a method to be used in tier 2 i.e., as follow-up on suspected irrelevant positive results from the standard in vitro test battery.
- 59 mix not required to identify pro-mutagens as xenobiotic metabolism is well reflected.
- HET-MN enables investigation of systemic availability of test compounds.

Organisation of pre-validation

- Project phases (Testing of coded compounds)

<table>
<thead>
<tr>
<th>Phase</th>
<th>No. of compounds</th>
<th>Lab 1</th>
<th>Lab 2</th>
<th>Lab 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6 (each in 3 labs)</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>6 (each in 2 labs)</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>8 (each in 2 labs)</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>IV</td>
<td>16 (each in 1 lab)</td>
<td>-</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
- Independent selection of coded compounds by Cosmetics Europe
- Independent coding & shipment of compounds: BfR, BioTeSys

Test Design

- Application on day 8 of chicken egg development
- Sampling on day 11
- Microscopical analysis of erythrocytes

Study design

1. Solubility study
2. Dose range finder: Max. dose 100 mg/egg, i.e., in line with max. dose in vivo.
3. At least 2 main experiments

Experimental design

- Solvent control: aqua DI, isopropyl myristate, 10% DMSO/EtOH
- Positive control: cyclophosphamide (pro-mutagen)
- At least three concentrations of test compound

Advantages of assay

- Not an animal experiment
- Clear intrinsic metabolic activity omits the need to add rat liver 59 mix
- Chicken eggs (SPF eggs used for vaccine production) globally available
- So far no difference observed between suppliers/strains

Results - pre-validation

- Read-out parameter
  - Evaluation of 6 eggs per dose group with 1000 cells per egg, i.e., 6000 cells per dose/control group
  - Micronucleus (MN) frequency in erythrocytes
  - Viability of eggs
  - Change of PCE/NCE ratio considered not sensitive enough
- Data evaluation
  1. Validity check
  2. Statistical analysis
  3. Consideration of biological relevance

Results - Xenobiotic Metabolism

- Phase I

<table>
<thead>
<tr>
<th>Tested</th>
<th>Substance</th>
<th>HET-MN Result</th>
<th>Involved CYP P450*</th>
<th>Confirmed by own data**</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Acetylaminofluorene (2AAF)</td>
<td>+</td>
<td>1A1/1A2</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>2-Aminoantracene (2AA)</td>
<td>+</td>
<td>1A2, 1B1</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>2,4-Diaminotoluene (2,4-DAT)</td>
<td>+</td>
<td>1A2</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>4-nitroquinoline 1 oxide (4-NQO)</td>
<td>+</td>
<td>NQO1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>+</td>
<td>1A1, 1B1</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>+</td>
<td>2B6, 2C9, 3A4</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Dimethylnitrosamine (DMN)</td>
<td>+</td>
<td>2B4, 2E1, 2A6</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Diethylnitrosamine (DNEA)</td>
<td>+</td>
<td>2A6</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>7,12-Dimethylbenz[a]anthracene</td>
<td>+</td>
<td>1B1, 1A1</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Iosfamide</td>
<td>+</td>
<td>2B1, 2B4, 2B5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>+</td>
<td>2E1</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Cytarabine</td>
<td>+</td>
<td>3A4</td>
<td>Daily, 3A4 mediated drug interactions</td>
<td></td>
</tr>
</tbody>
</table>

- Based on literature data, Additional testing underway

- Phase II

- Quantitative analysis of separate compartments, i.e., yolk sac membrane and developing liver.
- Clear enzyme activity of glutathione S-transferase, UDP-glucuronosyltransferase, sulfotransferase (data not shown) (N-acetyltransferases under investigation).
- Higher metabolic capacity in yolk sac membrane compared to liver due to greater size.
- Variability of enzyme activity - measured as pmol enzyme activity/min/mg protein - between the developing hen’s egg (own data) and the human system (literature) seems to be comparable to differences between mouse/rat (literature) and the human system.

HET-MN publications:

Wolf et al., 1997. Mutat Res 394: 163-175
Wolf et al., 2002. Mutat Res 514: 59-76
Wolf et al., 2008. Mutat Res 650: 150-164
Grewey et al., 2012, Mutat Res 747:118-34
Hothorn et al., 2013, Mutat Res 757: 68-78

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