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

Bertrand Desprez[1], Kerstin Reisinger[2], Dagmar Fieblinger[3], Andreas Heppenheimer[4], Jochen Kühnl[5], Juergen Kreutz[2], Manfred Liebsch[3], Andreas Luch[3], Katrin Maul[3], Albrecht Poth[4], Pamela Strauch[7], Wolfgang Voelkner[7], Thorsten Wolf[6], Ralph Pirow[3]

1 - Cosmetics Europe, Brussels, Belgium; 2 - Henkel AG & Co KGaA, Düsseldorf, Germany; 3 - German Federal Institute for Risk Assessment (BfR), Berlin, Germany; 4 - Harlan Cytotest Cell Research, Roßdorf, Germany; 5 -Beiersdorf AG, Hamburg, Germany; 6 - University of Osnabrueck, Osnabrueck, Germany; 7 - Envigo CRS GmbH, Roßdorf, Germany; kerstin.reisinger@henkel.com

## Abstract/introduction

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 S9 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.

## Conclusion

- **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.**
- **S9 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

- **Laboratories:** BfR, Envigo, Henkel.
- **Project phases (Testing of coded compounds)**

| Phase | No. of compounds | Lab 1 | Lab 2 | Lab 3 |
|---|---|---|---|---|
| I | 6 (each in 3 labs) | 6 | 6 | 6 |
| II | 6 (each in 2 labs) | 4 | 4 | 4 |
| III | 8 (each in 2 labs) | 5 | 5 | 6 |
| IV | 16 (each in 1 lab) | - | 8 | 8 |

- **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

***HET-MN mirrors systemic availability of compounds***
- Absorption
- Distribution via the vessel system
- Metabolism in the developing liver and the yolk sac membrane
- Excretion of metabolite into allantois (bladder equivalent)

- Not an animal experiment
- Clear intrinsic metabolic activity omits the need to add rat liver S9 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
- e.g. bioavailability check of test compound by modification of MN rate or vitality

(2) Statistical analysis
- Prediction model 1:lab-specific threshold /trend test
- PM 2:Umbrella-Williams-Test (Hothorn *et al.*, 2013)

(3) Consideration of biological relevance

| Parameter | Lab 1 | Lab 2 | Lab 3 | Overall |
|---|---|---|---|---|
| No. of chemicals | 14 | 20 | 23 | 32 |
| Sensitivity | 57 | 100 | 80 | 88 |
| Specificity | 100 | 100 | 92 | 97 |
| Accuracy | 79 | 100 | 87 | 92 |

## Results - Xenobiotic Metabolism

**Phase I**

| Tested | Substance | HET-MN Result | Involved CYP P450* | Confirmed by own data** |
|---|---|---|---|---|
| Coded | 2-Acetylaminofluorene (2AAF) | + | **1A1/1A2** | x |
| | 2-Aminoanthracene (2AA) | + | **1A2, 4B1, 1B1** | x |
| | 2,4-Diaminotoluene (2,4-DAT) | + | **1A2** | x |
| | 4-nitroquinoline 1 oxide (4-NQO) | + | **NQO1** | |
| | Benzo[*a*]pyrene | + | **1A1, 1B1** | x |
| | Cyclophosphamide | + | **2B6, 2C9, 3A4** | x |
| Non-coded in earlier project phases | Dimethylnitrosamine (DMN) | + | **2B4, 2E1, 2A6** | x |
| | Diethylnitrosamine (NDEA) | + | **2A6** | x |
| | 7,12- Dimethylbenz[*o*]anthracene | + | **1B1, 1A1** | x |
| | Ifosfamide | + | **2B1, 2B4, 2B5** | x |
| | Acrylamide | + | **2E1** | |
| | Cytarabine | + | Only **3A4**-mediated drug interactions | |

* 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., 2003. Food Chem Toxicol. 41:561-73
Wolf et al., 2008. Mutat Res 650: 150-164
Greywe et al., 2012, Mutat Res 747:118-34
Hothorn et al., 2013, Mutat Res 757: 68-78

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