*In vivo* genotoxic effects of dietary heme iron on rat colon mucosa and *ex vivo* effects on colon cells monitored by an optimized alkaline comet assay


Toxalim, Research Centre in Food Toxicology, Toulouse, FRANCE

ICAW 1-4 Sept 2015
Team
Genotoxicity and signalling

Team
Prevention and Promotion of Carcinogenesis by Food
Technical optimization of comet assays was performed, then used for \textit{in vivo} and \textit{in vitro} experiments.

Optimization: frozen samples, isolation of cells and throughput increase.

Example of application: heme iron diet (mimicking red meat) genotoxicity in rat colon mucosa.
Technical optimization: sample preparation

Colon mucosa cells collected by scraping (with KREPS buffer)
How to isolate cells from scraped colon mucosa?

Dounce + loose-fitting pestle
Sample preparation: how to isolate cells from scraped colon mucosa?

<table>
<thead>
<tr>
<th>Isolation step</th>
<th>20 up and down</th>
<th>40 up and down</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Buffer NaCl 75 mM, Na₂EDTA 24 mM)</td>
<td>Partial isolation Undamaged</td>
<td>Isolation complete Undamaged</td>
</tr>
</tbody>
</table>

Conditions are suitable for cell isolation
Sample preparation: how to freeze cells?

<table>
<thead>
<tr>
<th>Medium (isolation ± freezing)</th>
<th>Fresh cells</th>
<th>Cells after freezing/defreezing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NaCl 75 mM, Na&lt;sub&gt;2&lt;/sub&gt;EDTA 24 mM</strong></td>
<td>Undamaged</td>
<td><strong>NaCl 75 mM, Na&lt;sub&gt;2&lt;/sub&gt;EDTA 24 mM</strong></td>
</tr>
<tr>
<td><strong>DMEM + 10 % FCS</strong></td>
<td>Damaged</td>
<td><strong>DMEM + 10 % FCS + 10 % glycerol</strong></td>
</tr>
</tbody>
</table>

Conditions are suitable for freezing/defreezing.
Sample preparation: cells are still responsive to DNA damage

- Untreated cells = Negative control
- Cells treated with Ro 19-8022 (ex vivo) = Positive control (gift from Hoffmann-La Roche Ltd)

[comet + FpG (Formamidopyrimidine DNA glycosylase)]
Increase throughput: sample deposit

**Same Home-made Agarose**: 0.5% or 0.7% LMP

**Standard microscope slides**
- **Home-made coating**

**Superfrost standard microscope slides**
- Agarose:
  - 1 layer to include cells
  - 1 layer to protect the cells
  - Coverslip for agarose laying
- 1 deposit per slide

**20 wells Trevigen® slides**
- Slide already pre-coated

**20 wells Trevigen® slides**
- Slide pre-warming (37°C)
- Agarose: 1 layer to include cells, without coverslip
- 20 deposits per slide
Increase throughput: electrophoresis

Same alkaline comet assay protocol:
  Unwinding time: 40 min
  Home made buffer
  Electrophoresis tank
  Electrophoresis time: 24 min 0.8 V/cm

- Standard microscope slides
  - 3 slides performed /condition
  - 2 slides analysed
  - 24 slides/experiment
  - 8 conditions/experiment

- Trevigen® slides
  - 4 deposits performed /condition
  - 2 deposits analysed
  - 12 Trevigen® slides/experiment
  - 60 conditions/experiment
DNA staining and analysis

DNA staining: ethidium bromide + Coverslip

Standard microscope slides

Trevigen® slides

Analyses:
- Microscope Nikon 50i
- Komet 6.0 software
- Parameters determined: % DNA in tail, Tail length, Olive Tail Moment
- 50 cells/deposit analysed, 2 independent deposits

This protocol is suitable for middle throughput comet assays
Conclusions of technical optimizations: this protocol is suitable for further applications

Protocol optimized for:

- Freezing/defreezing of colon cells mucosa
- Mecanic cells isolation using a dounce

Throughput of the protocol increased using 20 wells Trevigen® slides (60 conditions instead of 8)
Examples of use of this optimized protocol

**In vivo studies**

**In vitro studies**
Background:
25% for men and 16% for women of colorectal cancer cases can be attributed to red meat intake (Perkins, 2011)

2007:
Colorectal cancer: A major cause of mortality in western countries
Environmental factors, particularly food

2011:
Epidemiological studies: Association between colorectal cancer and fresh red meat & cured-meat intake
Background: Heme iron is a major factor as described in carcinogenesis studies using carcinogen-initiated rats.

Catalysis: Heme → Lipoperoxidation → Aldehydes → 4-hydroxy nonenal → Carcinogenesis steps

Initiation: Normal Crypt → Aberrant Crypt

Promotion: Aberrant Crypt Focus (ACF) → Adenoma → Adenocarcinoma
Background: Heme iron intake increases promotion of colorectal carcinogenesis in rats

*Same letter are not significantly different
*Pierre et al., 2003

0 500 1000 1500 2000 2500

Heme in diet (µmol/g)

Aberrant Crypts
Foci

Mucin Depleted
Foci

**Pierre et al., 2004

0 1 2 3 4 5

MDF/colon

Same letter are not significantly different

*: significantly different from control diet without heme

Bastide et al., 2015

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Background: Heme-induced promotion of colorectal carcinogenesis is associated with an increase of genotoxicity measured by the number of:

- Anaphase bridges
- γH2AX positive cells

*Bastide et al., 2015*
The aim was to check if the heme-induced lipid peroxidation increase is responsible for the increment of genotoxicity in the colon rat mucosa of heme-fed rat.
In vivo study: Heme-induced fecal lipoperoxidation is associated with an increased genotoxicity (measured by comet assay)

Calcium = Iron chelation, Heme chelation

Unpublished data
From *in vivo* study to *in vitro* study

**Results from *in vivo* study:**

Heme diet → Catalysis → Lipo-peroxidation → Aldehydes → ??? → Genotoxicity (in vivo in colon mucosa)

**Aim of *in vitro* study:**

Heme diet → Catalysis → Lipo-peroxidation → Aldehydes (in fecal water) → ??? → Genotoxicity (in vitro on colonic cells)
In vitro study: are aldehydes responsible for heme-induced genotoxicity?

Obtention of « fecal water » using mecanic aqueous extraction of feces from rats exposed to different diets = To obtain biodisposable fraction of feces

For treatment of murine normal colonic epithelial cells
To evaluate genotoxic activity
**In vitro study: aldehydes from heme-induced lipoperoxidation are responsible for increased genotoxicity**

4 fecal water:
- **CON** = Control
- **CON + resin** = less aldehydes (using polymer beads to bind aldehydes)

**Fecal water**
- **HEM** = Heme
- **HEM + resin**

Unpublished data

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**Murine colonic epithelial cells (Pierre et al., 2007)**

**Flowchart:**
- **Heme diet** → **Catalysis** → **Lipoperoxidation** → **Aldehydes (in fecal water)** → **Genotoxicity**
Conclusions

Heme → **Catalysis** → **Aldehydes** production via lipoperoxidation

**In vivo results**

**Genotoxicity**

→ **In vitro results**

→ Promotion of colorectal carcinogenesis And/or initiation
Prospects

Automatic acquisition of images

Manual Microscope
Nikon 50i

Motorized Microscope
Nikon NiE

But semi-automatic quantification of comet to exclude such.....

.....artefacts
In summary, we optimized and used alkaline comet assay protocol

Introducing a freezing step and mecanic cells isolation

Increasing throughput adapting standard protocol using Trevigen® slides

Applied to our research field about heme-iron diet

Can be applied to other type of cells, samples and research fields