CYTOTOXICITY AND ITS IMPACT ON THE IN VIVO COMET ASSAY

by Marie Z. Vasquez
Helix3 Inc.

CYTOTOXICITY

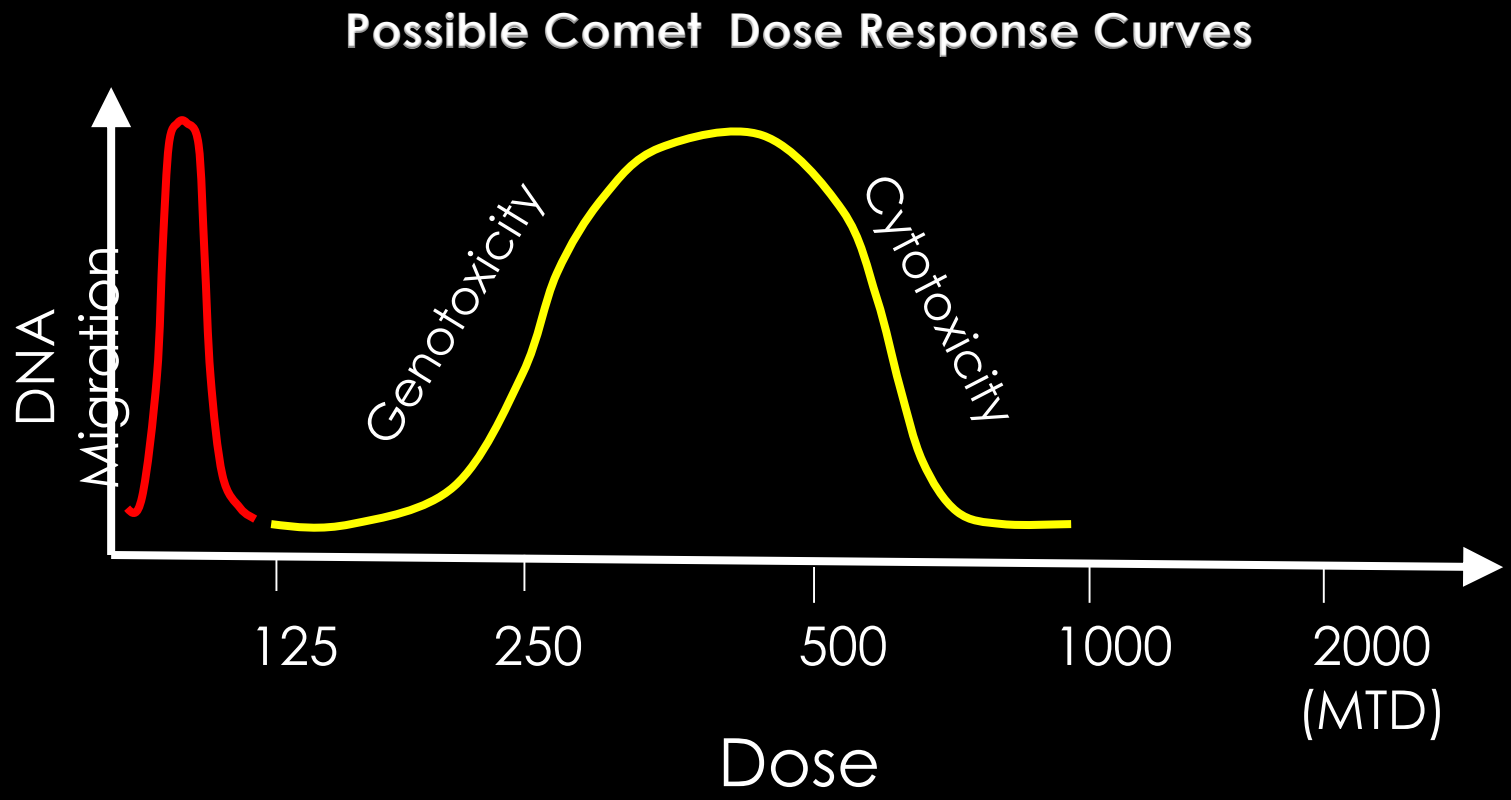
- Definition
  - Toxicity vs. Cytotoxicity
  - Pre-Lethal vs. Post-Lethal Effects
- Measurements
  - Post lethal
  - Pre-lethal
- Impact
  - Acute vs. Subacute
  - Vehicle / Site of Contact
  - Tissue / Cell Selection

DEFINITION

- Toxicity vs. Cytotoxicity
- Pre-Lethal vs. Post-Lethal Effects

DEFINITION

Toxicity vs. Cytotoxicity



The genotoxic and cytotoxic responses in tissues are often well below the MTD

DEFINITION

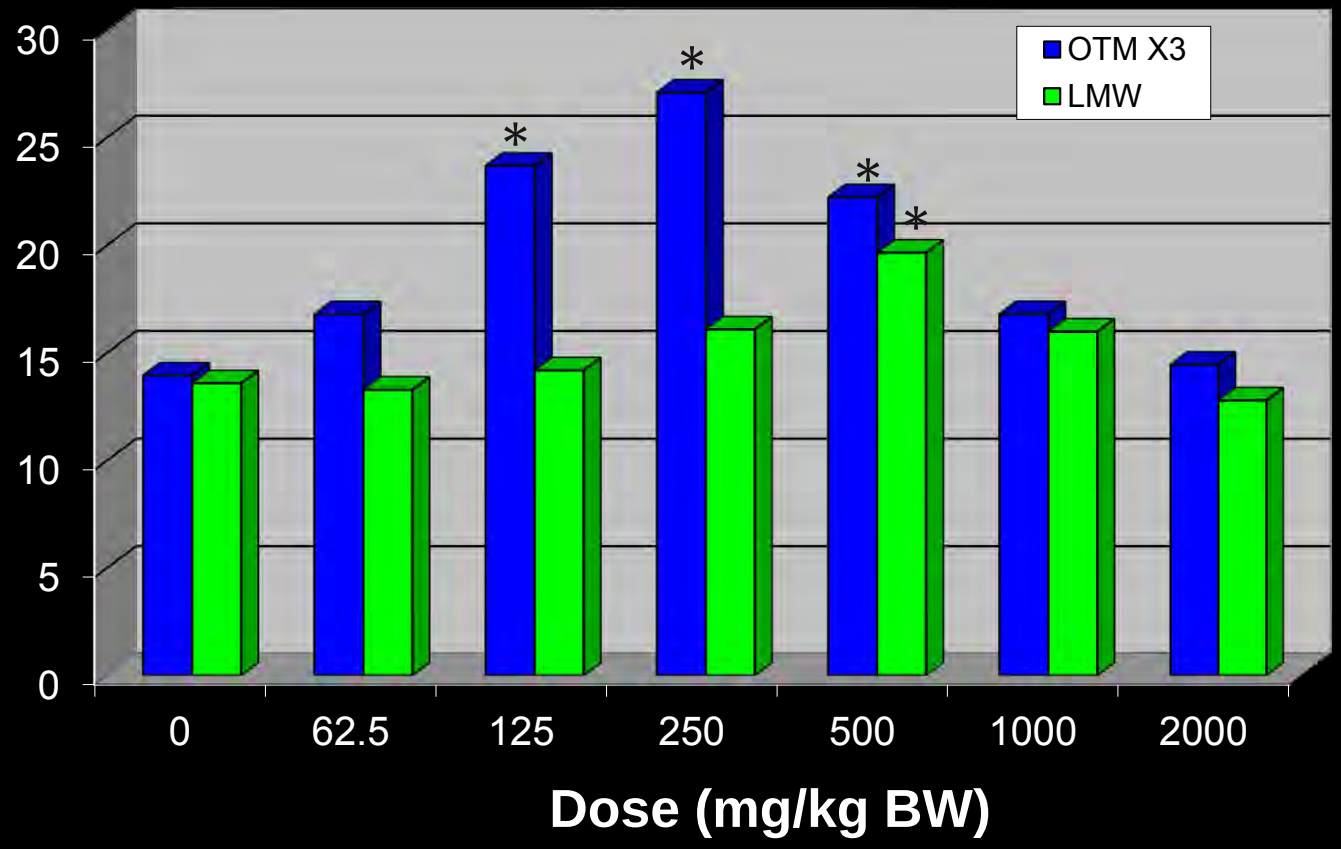
Toxicity vs. Cytotoxicity

- Case Study 1:
  - Non-Toxic Compound in the Colon
    - 3 daily oral doses at 0, 62.5, 125, 250, 500, 1000, and 2000 mg/kg
    - 5 animals per dose
    - Sampled at 4 hr

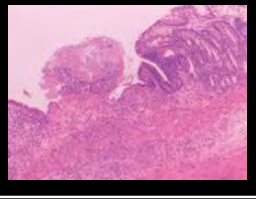
DEFINITION

Toxicity vs. Cytotoxicity

Results:



- Mucosa loss
- Inflammatory granulation tissue substitutions
- Edema



DEFINITION

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Toxicity vs. Cytotoxicity Summary

- Significant cytotoxicity can exist in the absence of conventional signs of toxicity (e.g. lethality) and well below the MTD

- To ensure that a genotoxic dose response can be adequately detected, the MTD for comet should be based on more sensitive measurements of cytotoxicity

DEFINITION

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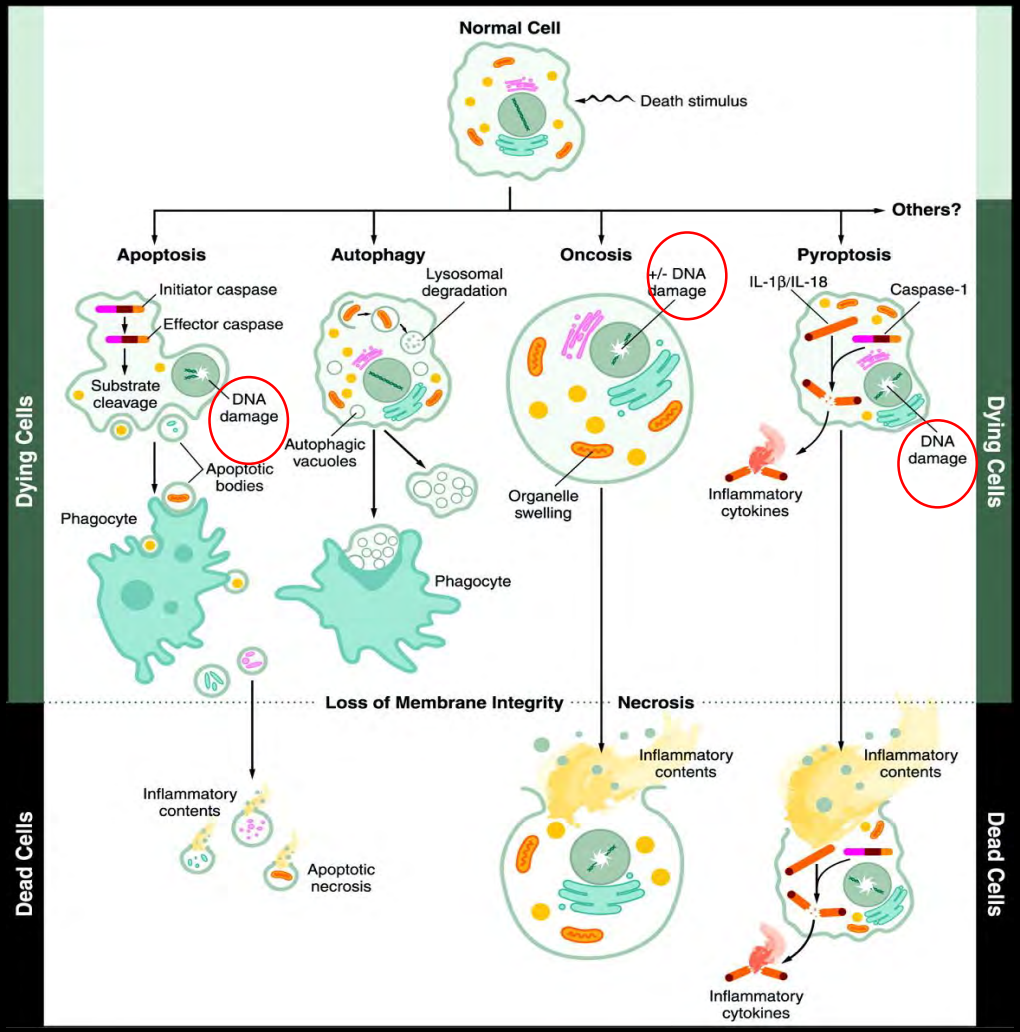
Pre-Lethal vs. Post-Lethal Effects



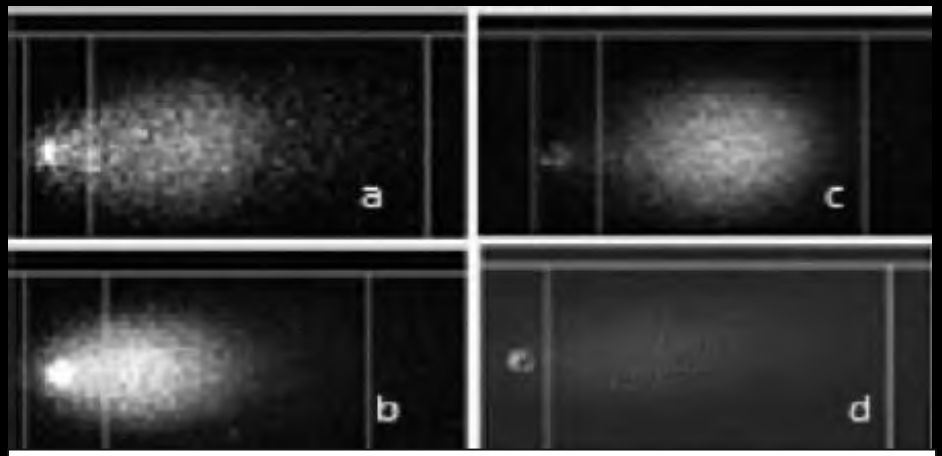
Pre-lethal events that occur before necrosis /apoptosis is evident are most likely to increase DNA migration

Adapted from Fink, S.J. and Cookson, B.T. Infection and Immunity (2005) 1907-

DEFINITION

Pre-Lethal vs. Post-Lethal Effects

- "Hedgehogs" or "ghost cells" are cells with extreme damage–they are NOT dead/dying cells
- LMW DNA fragments from dead/dying cells migrate out of gels during electrophoresis



Acellular nuclear DNA exposed to EMS: (a) and (b) are "hedgehogs" and (c) and (d) are "ghost cells"
Vasquez, MZ. Mutation Research 2012

REVIEW

The Comet Assay for DNA Damage and Repair

Principles, Applications, and Limitations

Andrew R. Collins*

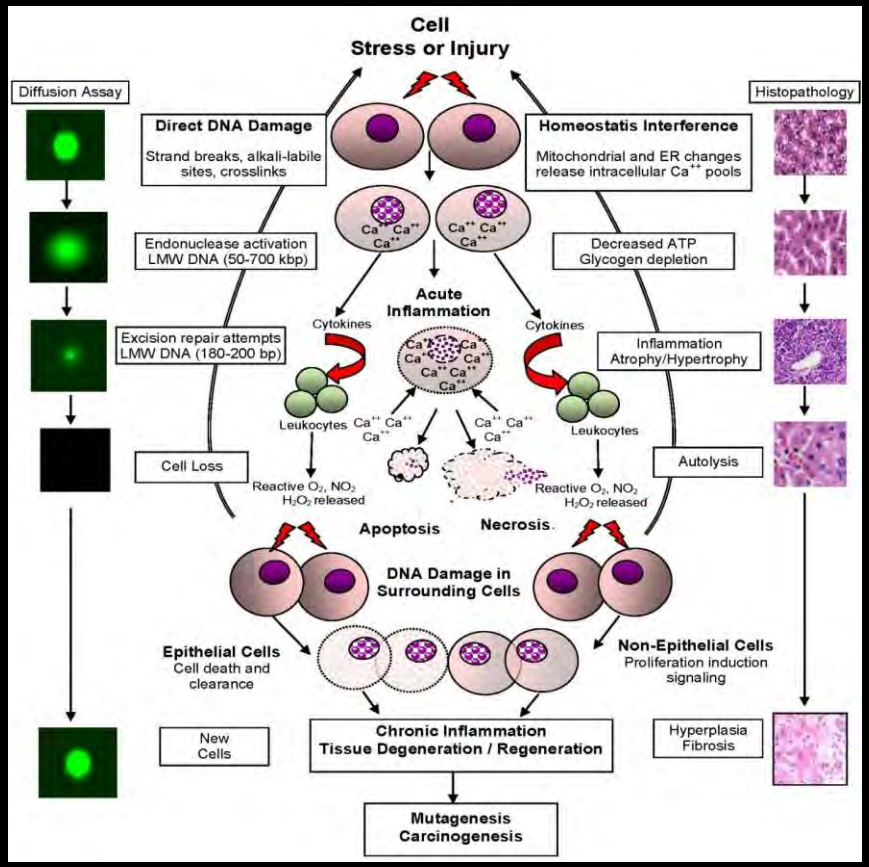
Environmental and Molecular Mutagenesis 41:260–269 (2003)

Detection of Ghost Cells in the Standard Alkaline Comet Assay Is Not a Good Measure of Apoptosis

Sophie Meintières,[1] Fabrice Nesslany,[1] Marc Pallardy,[2] and Daniel Marzin[1,3]*

DEFINITION

Pre-Lethal vs. Post-Lethal Effects



DEFINITION

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Pre-Lethal vs. Post-Lethal Effects Summary

- Cytotoxicity-related DNA strand breaks that occur before necrosis/apoptosis is histologically evident can result in a “false positive”
- Baseline levels of DNA migration caused by the loss of damaged cells to necrosis/apoptosis can result in a “false negative”
- The continuum of post-necrotic tissue inflammation and regeneration can also result in a “false positive”

DEFINITION SUMMARY

- The definition of cytotoxicity should include both the pre- and post-necrotic/apoptotic stages in cell injury that can increase DNA migration

- All of these stages should be identified/measured in comet studies and addressed in the interpretation of results

- To ensure that a genotoxic dose response can be adequately detected, pre-lethal endpoints may be the best method for selecting the appropriate doses

MEASUREMENTS

- Post-Lethal
- Pre-Lethal

MEASUREMENTS

Post-Lethal

Case Study 2:

- Thioacetamide

  - Non-genotoxic carcinogen tested in the liver
  - Single IP dose at 0, 50, 100 and 200 mg/kg
  - 5 animals per dose
  - Sampled at 1, 3, 6, and 24 hrs

MEASUREMENTS

Post-Lethal

Endpoints Collected:

- Comet
- ALT , AST, and Glutamate dehydrogenase (GLDH)
- Histopathology (caspase III; TUNEL)

MEASURMENTS

Post-Lethal

Comet Results:

- Statistically significant dose response increase in OTM at 1 and 24 hrs
- No increase at 3 or 6 hrs

1 hr

Group	N	Mean	Standard Deviation	Ratio to Reference	Trend p-Value	Pairwise p-Value
0 mg/kg	10	0.05	1.81	REF	REF	REF
50 mg/kg	10	0.08	1.99	0.87	NF	0.352
100 mg/kg	10	0.06	1.73	0.97	0.355 -	0.966
200 mg/kg	10	0.10	1.75	0.77	0.045 *	0.041 *

24 hr

Group	N	Mean	Standard Deviation	Ratio to Reference	Trend p-Value	Pairwise p-Value
0 mg/kg	10	0.06	1.82	REF	REF	REF
50 mg/kg	10	0.08	2.70	0.86	0.130 -	0.532
100 mg/kg	10	0.13	2.52	0.70	0.011 - *	0.059
200 mg/kg	10	0.15	1.99	0.65	0.005 **	0.025 *

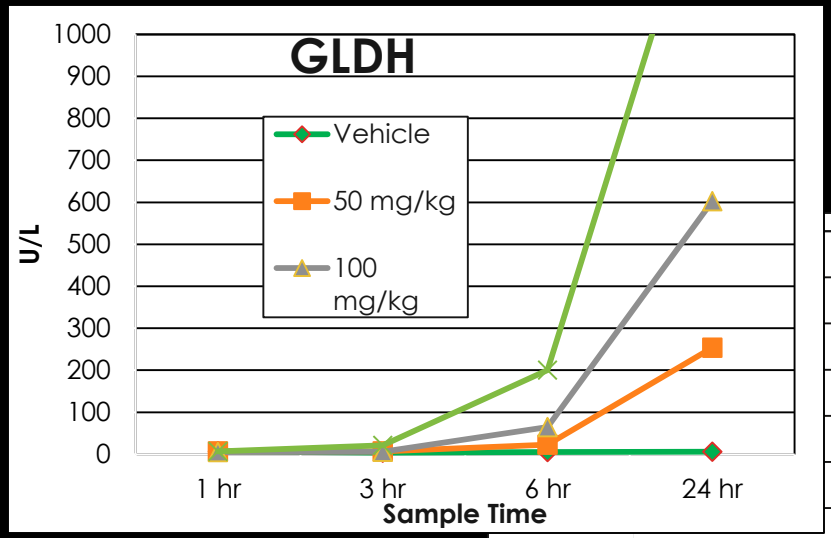
REF: Denotes group used as reference in the statistical tests and ratio calculations.
Sign (+, -) attached to the trend p-value indicates direction of trend test; no sign indicates two-sided test.
NF: Denotes no follow up test required. ND: Denotes not included in test.
* Statistically significant at 0.05 level ** Statistically significant at 0.01 level

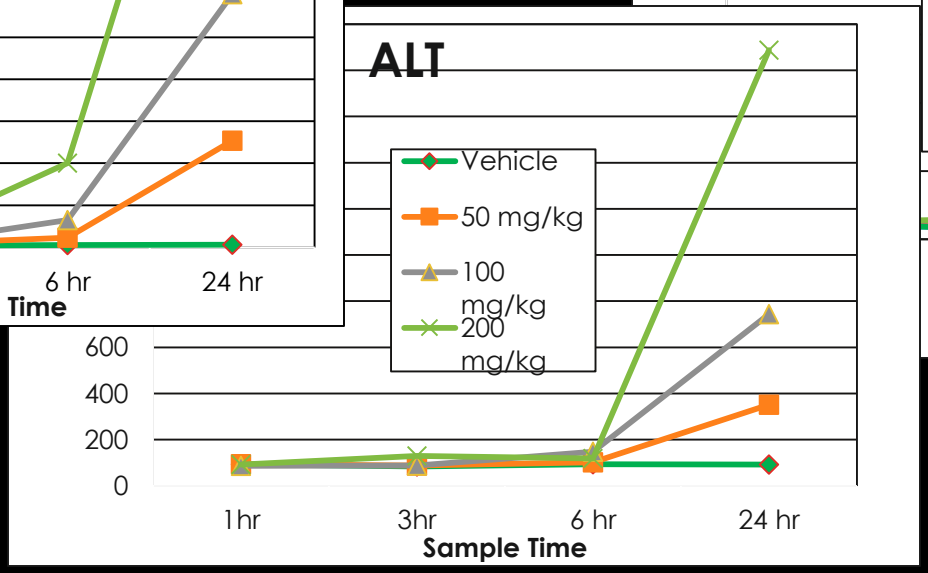
MEASUREMENTS

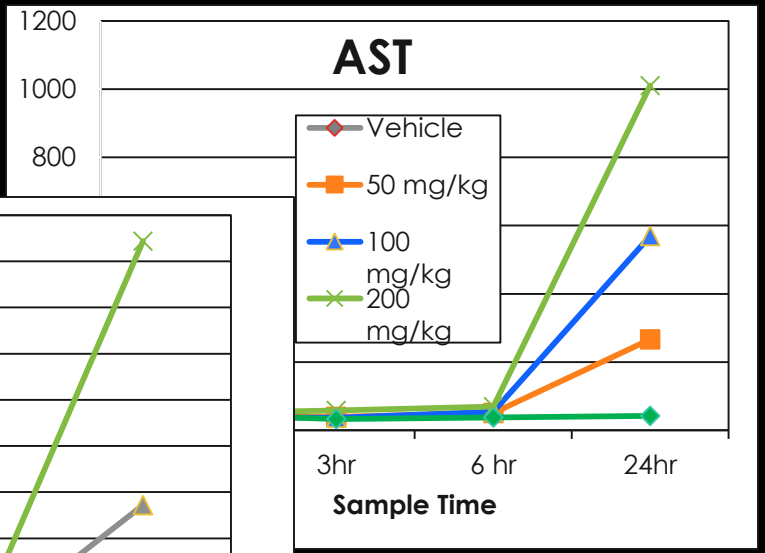
Post-Lethal

Blood Clinical Pathology Results:

- Marked GLDH increase by 6 hours
- GLDH, ALT and AST increased at 24 hours





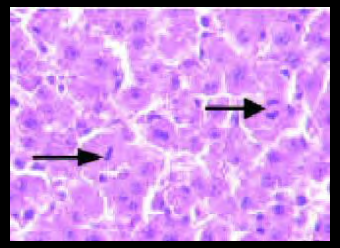


MEASUREMENTS

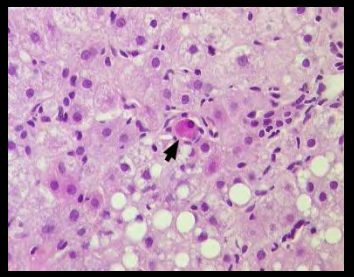
Post-Lethal

Liver Pathology Results:

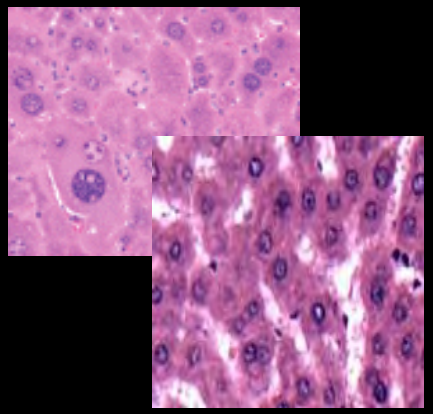
- 3 hrs: Increased mitosis



- 6 hrs:
  - Single cell and centrolobular hepatocellular necrosis
  - Cleaved Caspase at 50 mg/kg



- 24 hrs:
  - Hepatocellular hypertrophy
  - glycogen depletion



MEASUREMENTS

Post-Lethal

Case Study 2 Conclusions:

- Increased DNA migration was the earliest and only detectable effect at 1 hr
- At 3 and 6 hrs, increased mitosis and single cell necrosis were concordant with baseline levels of DNA migration
- Increased ALT, AST, GLDH, hypertrophy and glycogen depletion at 24 hrs indicate that post-necrotic events can contribute to DNA migration

MEASUREMENTS

Post-Lethal

Case Study 3:

Contents lists available at SciVerse ScienceDirect
Mutation Research/Genetic Toxicology and Environmental Mutagenesis
journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

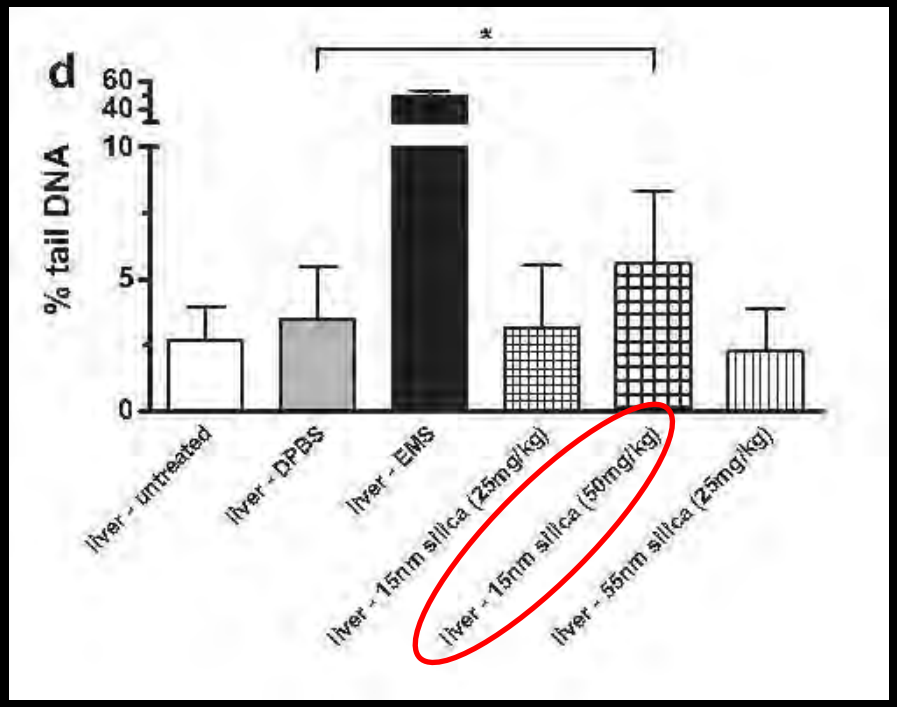
Silica nanoparticles administered at the maximum tolerated dose induce genotoxic effects through an inflammatory reaction while gold nanoparticles do not

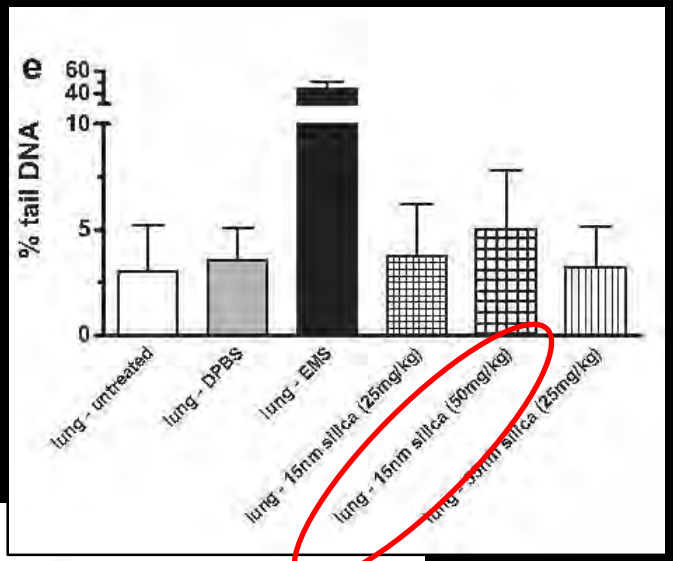
Thomas R. Downs[a], Meredith E. Crosby[a,1], Ting Hu[a], Shyam Kumar[b], Ashley Sullivan[a], Katherine Sarlo[a], Bob Reeder[a], Matt Lynch[a], Matthew Wagner[a], Tim Mills[c], Stefan Pfuhler[a,*]

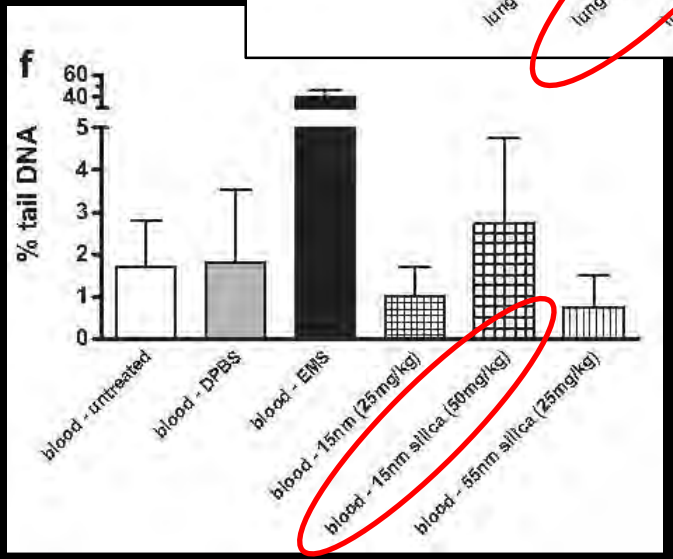
- Silica Nanoparticles (15 nm) in liver, lung and blood
- 3 daily I.V. doses at 0, 25, 50 mg/kg
- 5 animals per dose
- Sampled at 4 hr

MEASUREMENTS

Post-Lethal



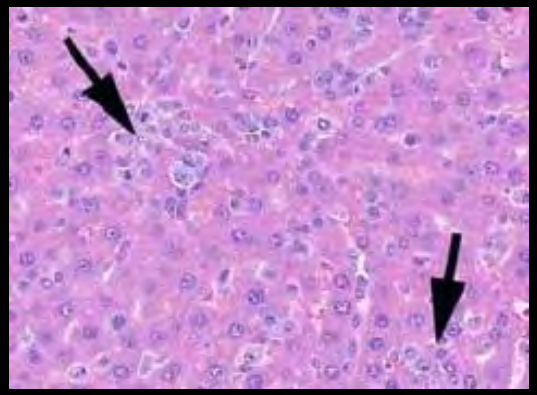




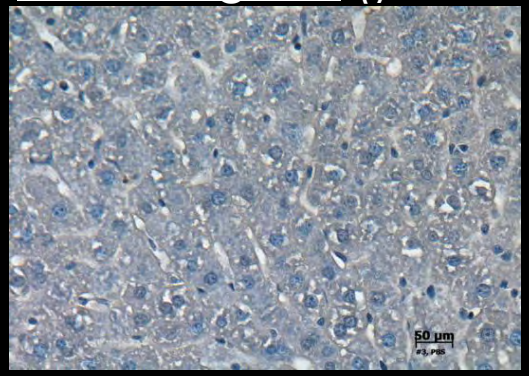
MEASUREMENTS

Post-Lethal

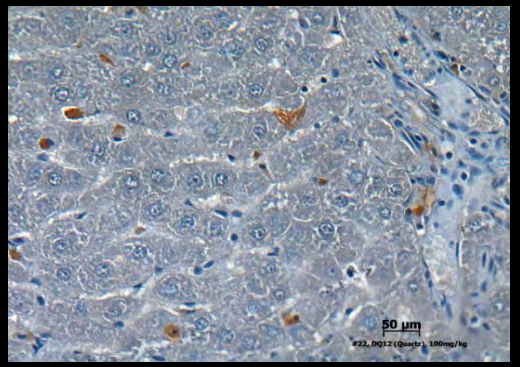
Liver histopathology (H&E stain, left two images) reveals neutrophil infiltration (arrows), hemorrhage (H) and tissue necrosis, and increased occurrence of mitotic figures (yellow circles)



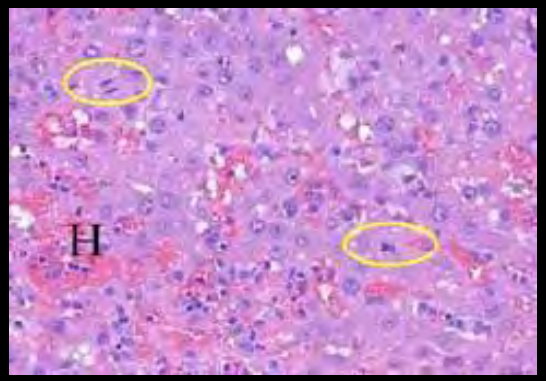
15nm silica, 50 mg/kg bw



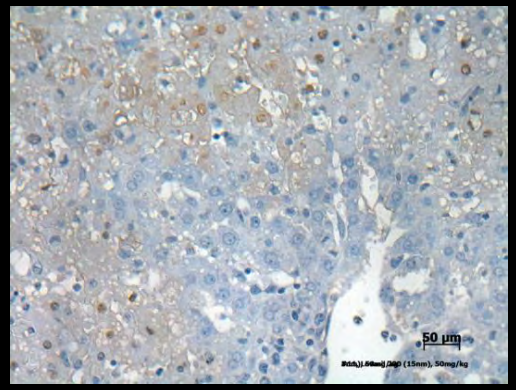
Control



DQ12 Quartz



15nm silica, 50 mg/kg bw



15nm silica, 50 mg/kg bw

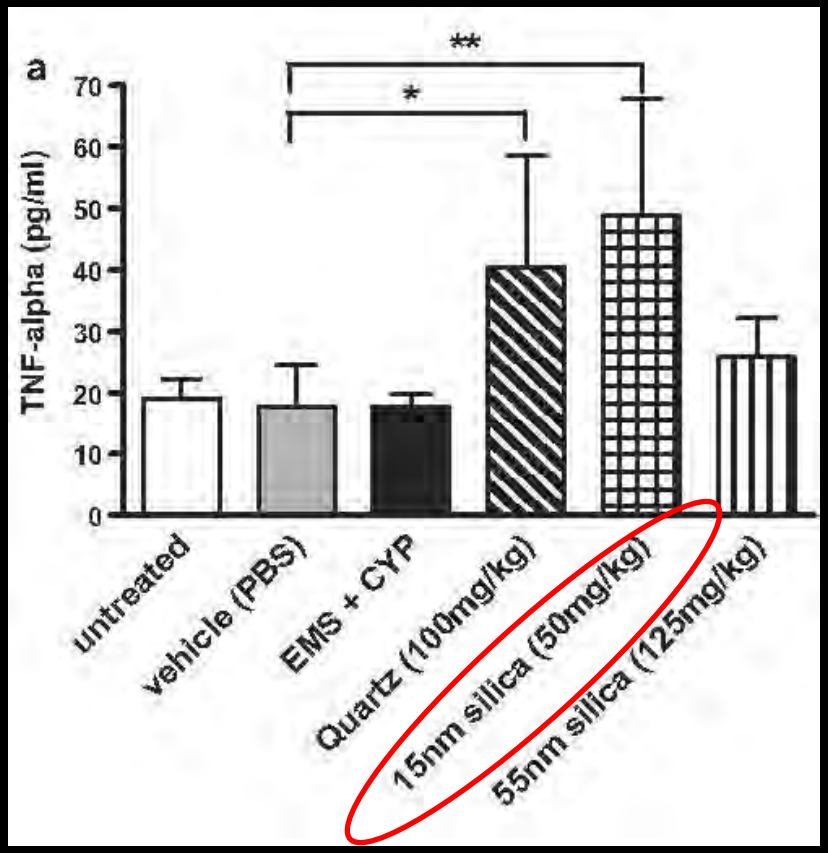
DeadEnd TUNNEL assay - Brown spots = Apoptotic nuclei

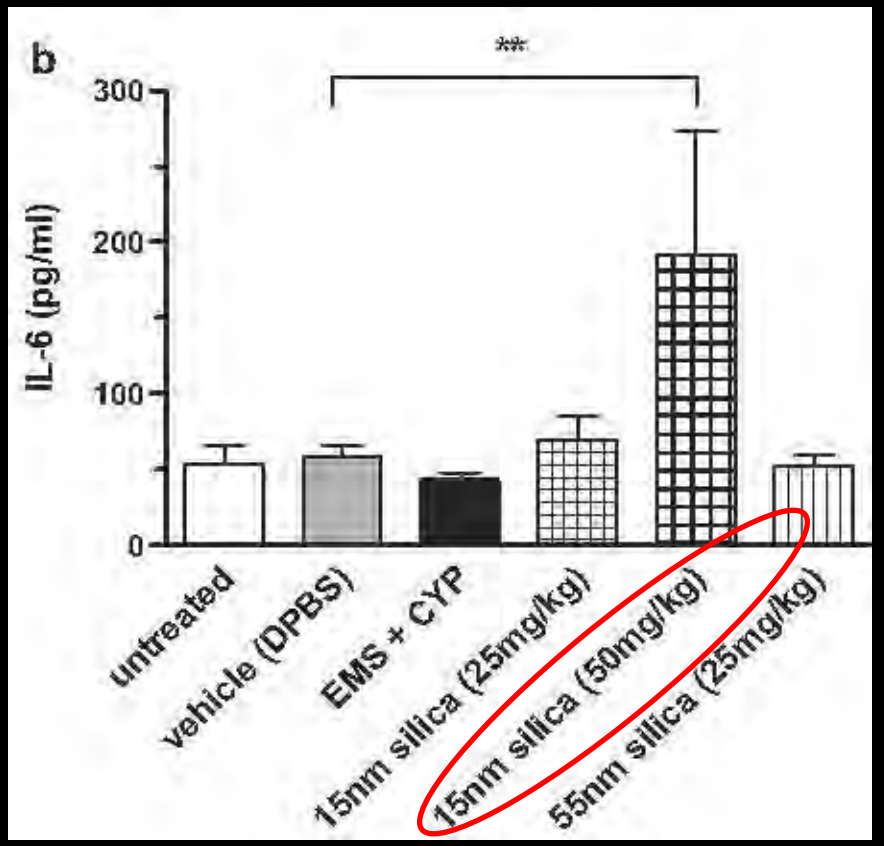
Liver damage followed by inflammatory response

MEASUREMENTS

Post-Lethal

Additional data:





MEASUREMENTS

Post-Lethal

Case Study 3 Conclusions:

- Minimal to moderate inflammation indirectly induces DNA damage through the release of cell-derived oxidants

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MEASUREMENTS

Post-Lethal Summary

- Significant to severe necrosis may not be associated with increased DNA migration due to the loss of dead/dying cells during processing

- The following post-necrotic/apoptotic endpoints may be the best cytotoxicity measurements for *in vivo* comet studies:
  - Glycogen depletion
  - Increased ALT/AST/GLDH
  - Hypertrophy / hyperplasia
  - Minimal to moderate inflammation

MEASUREMENTS

Pre- Lethal

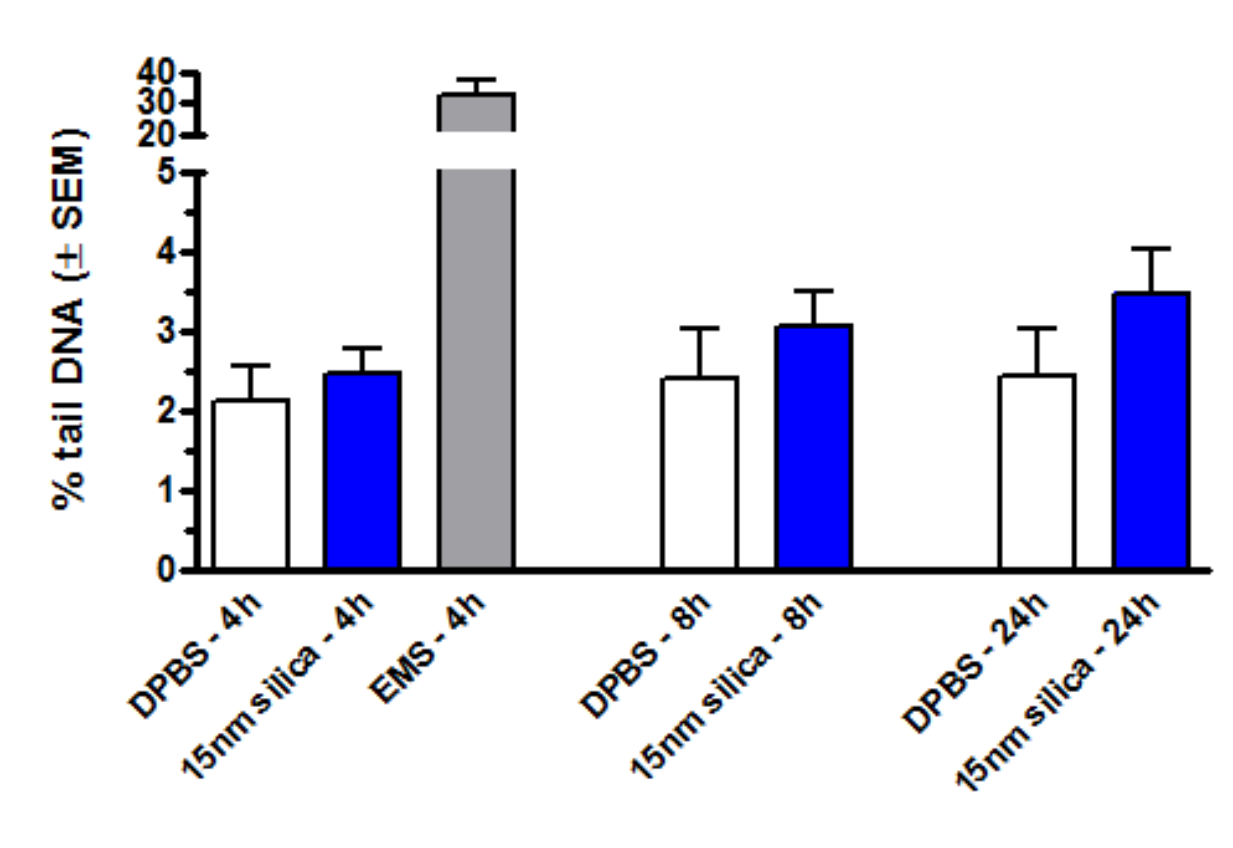
Case Study 3 (Continued):

- 15 nm Silica nanoparticle
- Single i.v. injection
- 24hr sample time

MEASUREMENTS

Pre- Lethal

Results:



Slight increase, increasing with time

Measurements
Pre- Lethal

Animal Number:	25	26	36	37	38	40	29	30	31	32	33	34	35
Treatment:	Vehicle (PBS)		Levasil 200 (5 mg/kg)				Levasil 200 (50 mg/kg)						
Liver Findings													
Infiltrate, mononuclear cell	-	-	1	1	-	1	2	2	1	2	2	2	2
Infiltrate, neutrophilic, portal	-	-	-	-	-	-	1	1	1	1	1	1	1
Infiltrate, neutrophilic, sinusoids	-	-	-	-	-	-	1	1	1	1	1	1	1
Increased mitotic figures, Kupffer cells	-	-	-	-	-	-	1	1	-	1	1	1	1

Histopathological findings

single i.v injections, 24h time point

Mild mononuclear infiltration

1 = Minimal, **2 = Mild**, **3 = Moderate**, 4 = Marked; - = Finding not present.

Measurements
Pre- Lethal

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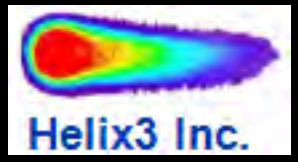
Case Study 3 Conclusions:

- Extreme doses of the silica NPs increased DNA migration 2-fold with 3 daily admins
- Single i.v. injection of the same material/dose lead to small but reproducible increase
- Histological findings were ‘mild’ or minimal’ at most
- Effects observed only at MTD, associated with liver toxicity and an inflammatory response involving oxidative stress pathways

MEASUREMENTS

Pre- Lethal

Case Study 4:



- EMS
- Genotoxic and cytotoxic carcinogen in the liver
- 3 daily oral doses at 0, 25, 50, 100 mg/kg
- 5 animals per dose
- Sampled at 4 hrs

MEASUREMENTS

Pre- Lethal

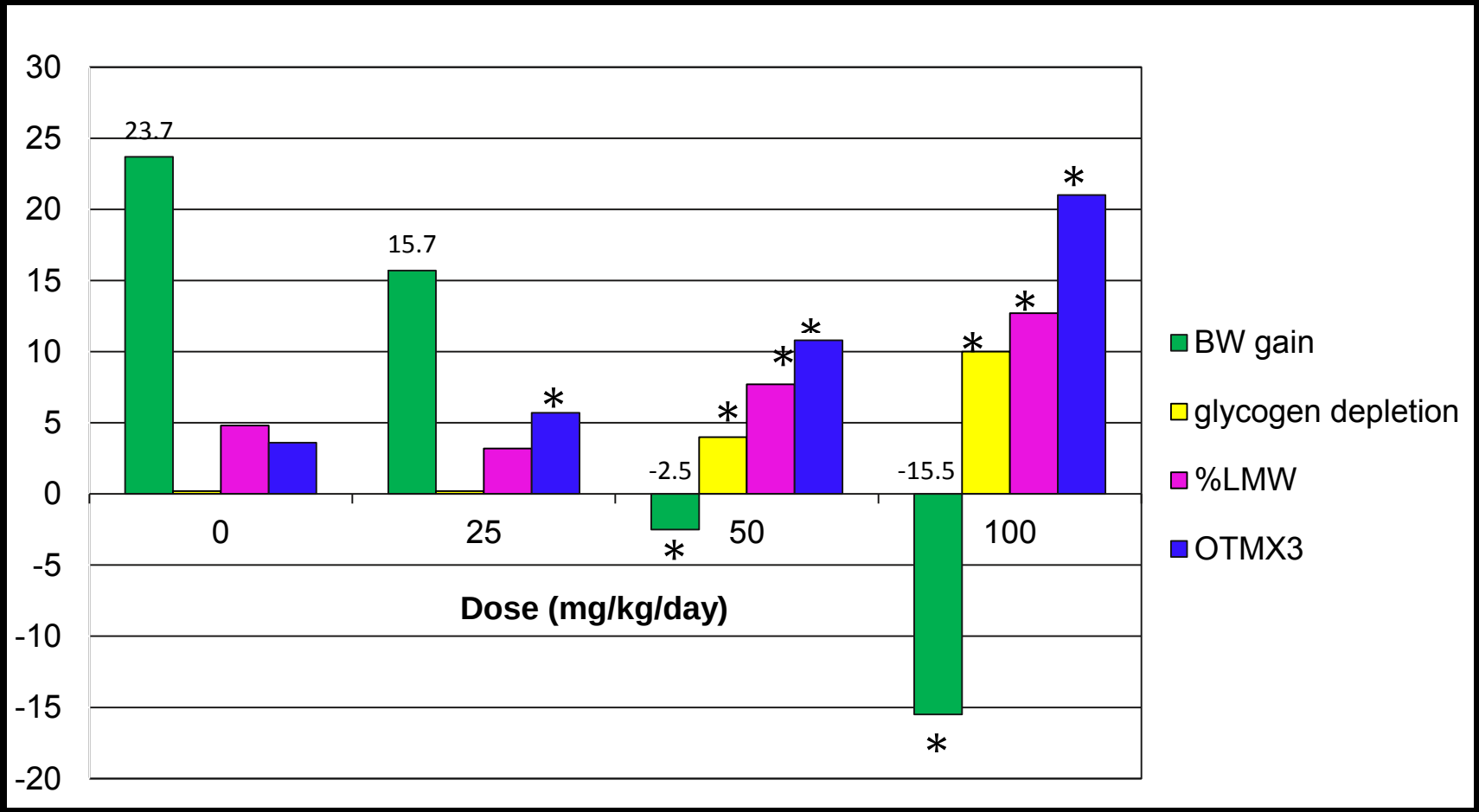
Endpoints Collected:

- Comet
- LMW DNA Diffusion
- Body Weight Gain
- Histopathology

MEASUREMENTS

Pre- Lethal

Results:



MEASUREMENTS

Pre- Lethal

Case Study 4 Conclusions:

- Significant changes in %LMW, glycogen depletion and body weight gain indicate a possible cytotoxic influence on DNA migration at doses ≥50 mg/kg

MEASUREMENTS

Pre- Lethal Summary

- The following pre-necrotic/apoptotic endpoints may be the best cytotoxicity measurements for *in vivo* comet studies:
  - Minimal to moderate inflammation
  - Glycogen depletion (liver only)
  - Significant (≥10%) decrease in body weight gain
  - Significant ($p<0.05$) increase in %LMW

MEASUREMENTS SUMMARY

- Histological evaluation for necrosis/apoptosis is insufficient for determining the potential influence of cytotoxicity on DNA migration

- Endpoints that may be detected early:
  - Minimal-moderate inflammation / hypertrophy
  - LMW DNA diffusion
  - glycogen depletion

  or more quantitatively
  ALT/AST
  - IL-6/TNFa
  - Decreased body weight gain

  may be more indicative of comet-relative cytotoxicity

IMPACT

- Acute vs. Subacute
- Tissue / Cell Selection

IMPACT
ACUTE VS. SUBACUTE

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Case Study

Mutation Research 702 (2010) 40–69

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Community address: www.elsevier.com/locate/mutres

Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies

Andreas Rothfuss[a,*], Mike O'Donovan[b], Marlies De Boeck[c], Dominique Brault[d], Andreas Czich[e], Laura Custer[f], Shuichi Hamada[g], Ulla Plappert-Helbig[h], Makoto Hayashi[k], Jonathan Howe[i], Andrew R. Kraynak[j], Bas-jan van der Leede[c], Madoka Nakajima[k], Catherine Priestley[b], Veronique Thybaud[d], Kazuhiko Saigo[l], Satin Sawant[m], Jing Shi[n], Richard Storer[j], Melanie Struwe[o], Esther Vock[p], Sheila Galloway[j]

IMPACT
ACUTE VS. SUBACUTE

Comet Study Design:

- 3 or 29 daily oral dose administrations
- Doses: 0, 600, 1200 mg/kg/day (3 Days) or 0, 160, 300, 600 mg/kg/day (29 Days)
- Sample time: 3 hrs
- Tissues: Liver and stomach

IMPACT
ACUTE VS. SUBACUTE

Liver Comet

Gemifloxacine mesylate

Study length	Doses (mg/kg bw/day)	Group mean %TI (±SD)	Fold Increase (Mean)
3 days	0	1.29 ± 0.64	1
	600	1.30 ± 0.40	1
	1200	2.28 ± 1.20**	1.77
	EMS	4.65 ± 0.11**	3.59
29 days	0	0.93 ± 0.36	1
	160	0.80 ± 0.34	0.86
	300	0.71 ± 0.12	0.76
	600	0.85 ± 0.16	0.91
	EMS	6.59 ± 0.33**	31.59

Reported Comet Result: Positive at 3 days, but Negative at 29 days

IMPACT
ACUTE VS. SUBACUTE

European Medicines Agency
Pre-authorisation Evaluation of Medicines for Human Use

London, 19 March 2009

WITHDRAWAL ASSESSMENT REPORT
FOR
FACTIVE

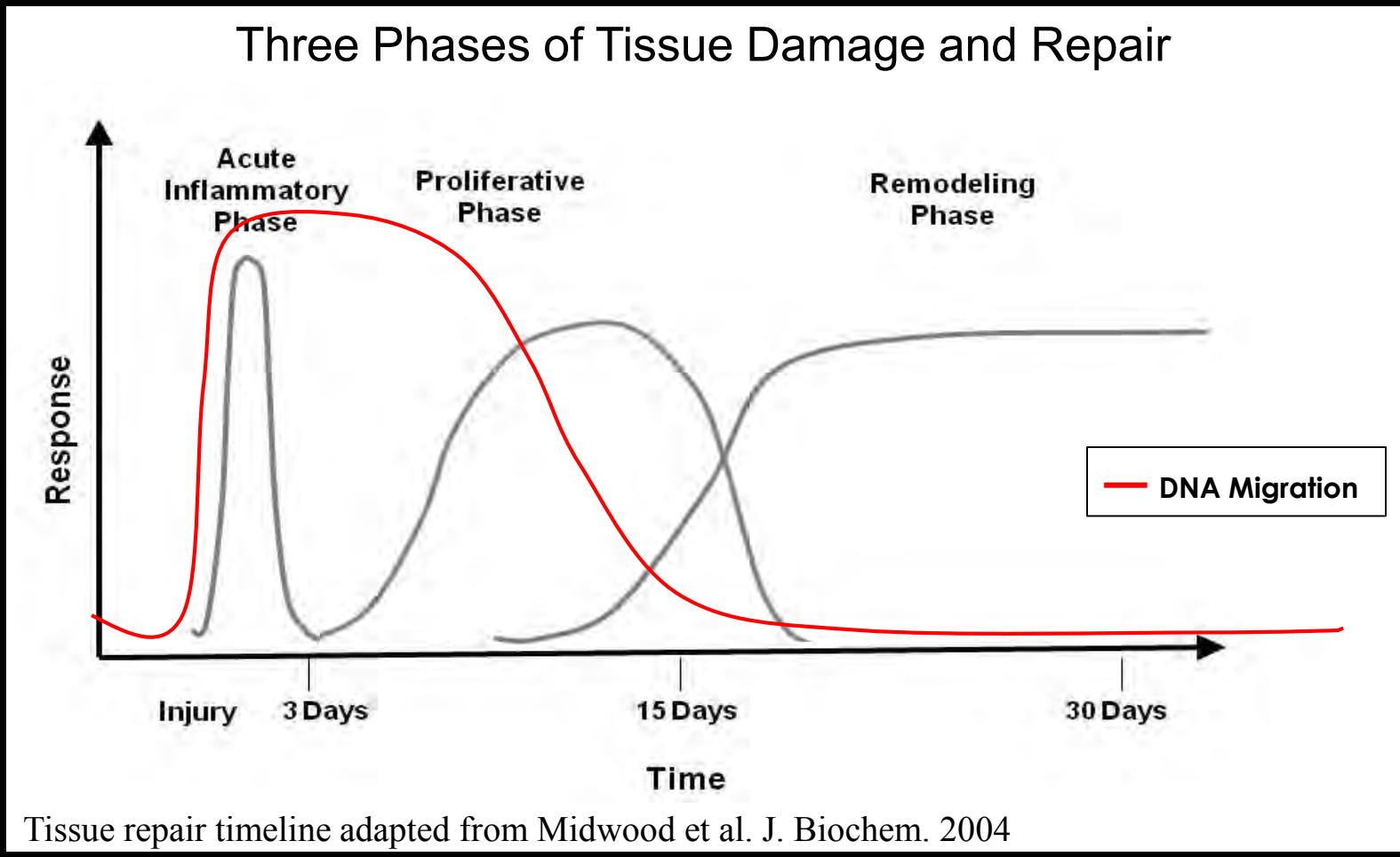
International Nonproprietary Name:
Gemifloxacin

Procedure No.EMEA/H/C/995

IMPACT
ACUTE VS. SUBACUTE

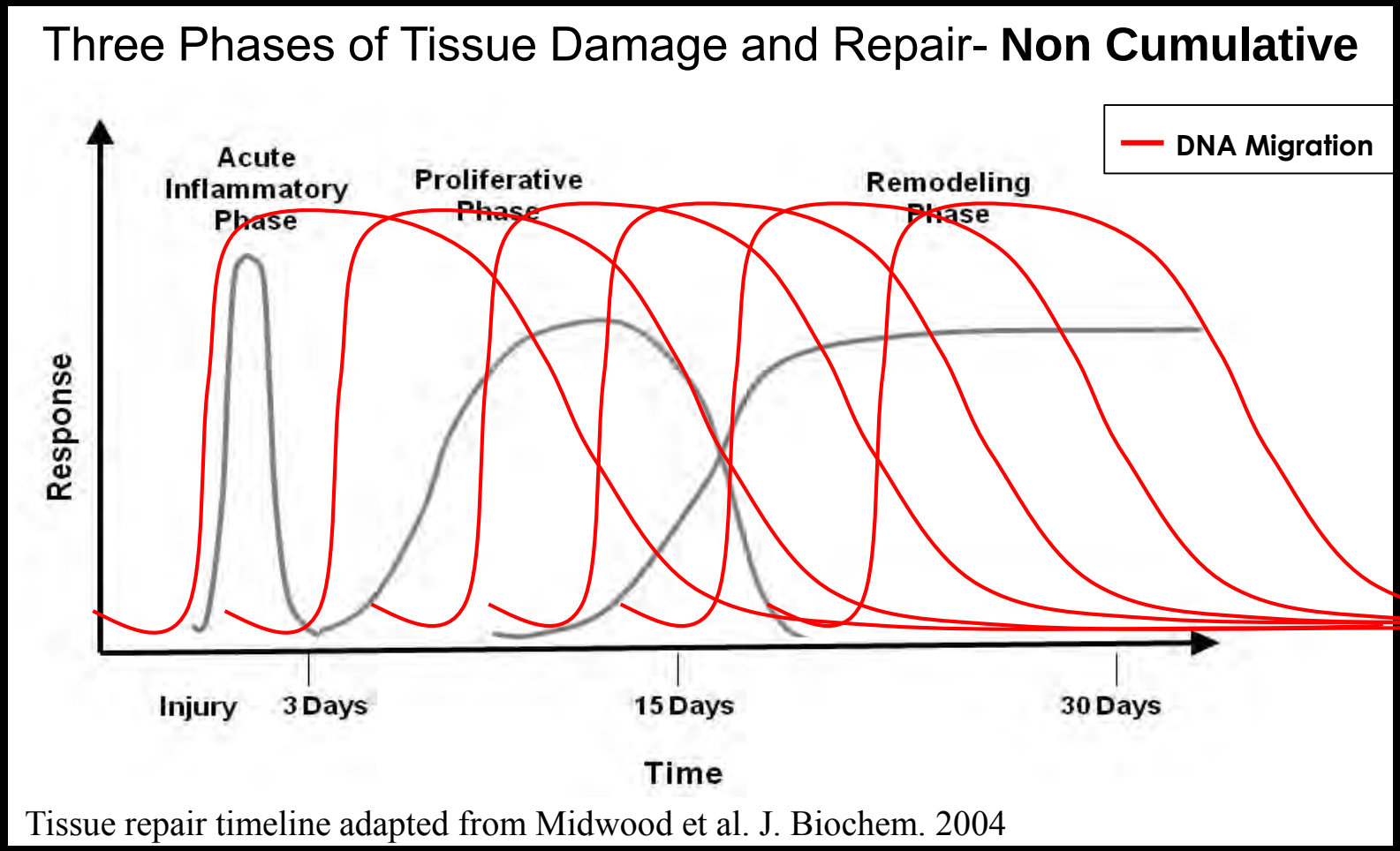
- EMEA Conclusions:
- Kidney is the rat target organ of toxicity leading to inflammation and tissue damage at 600 mg/kg
- Hepatotoxic effects are only evident in dog
- Tmax = 0.5-2 hrs with majority excreted unmetabolized after 24 hrs
- Human treatment duration: 5 days
- No evidence of systemic accumulation with repeat doses
- Helix3 Conclusions: Study design in collaborative study was inappropriate for gemifloxacine

IMPACT
ACUTE VS. SUBACUTE



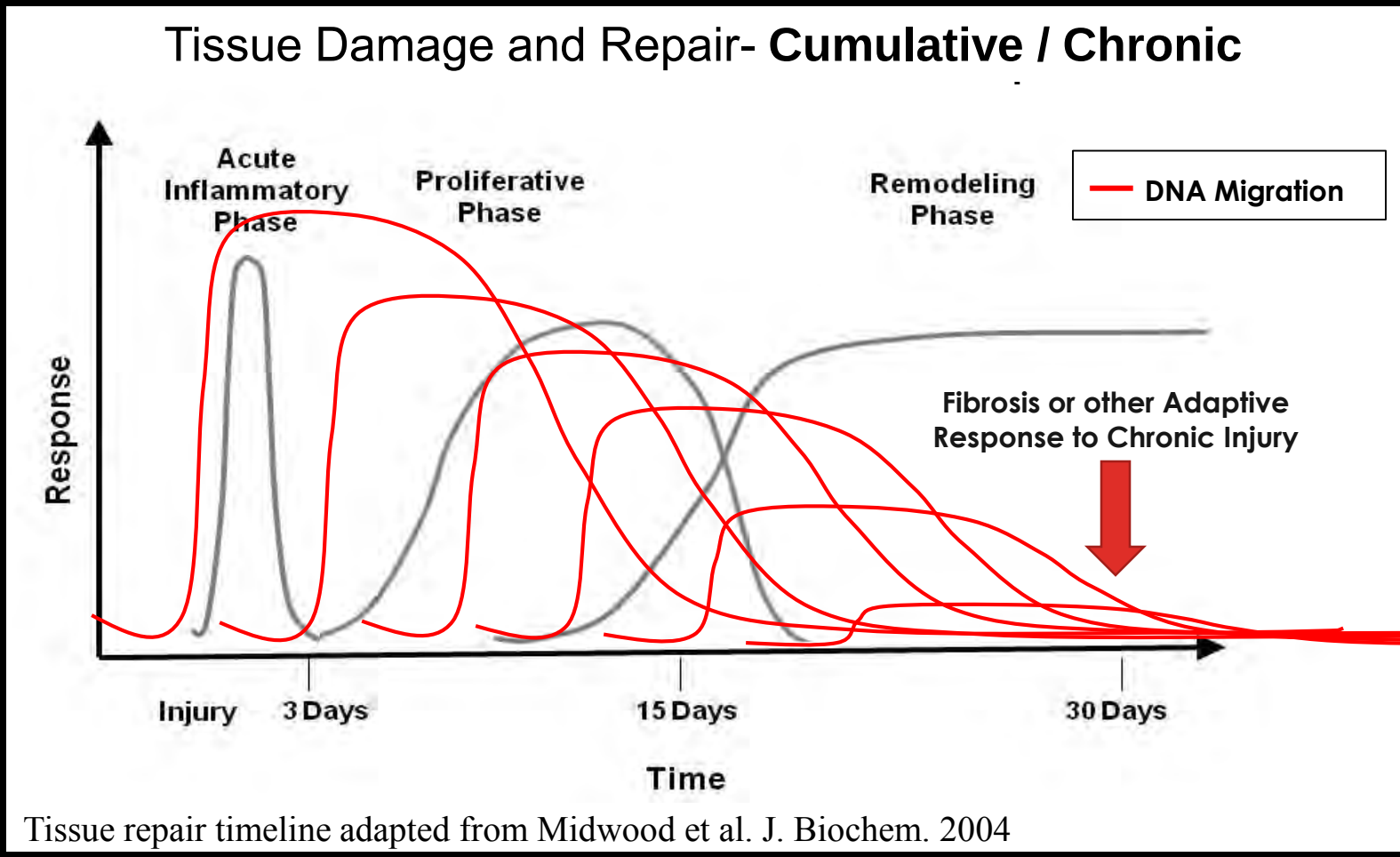
Tissue repair timeline adapted from Midwood et al. J. Biochem. 2004

IMPACT
ACUTE VS. SUBACUTE



Tissue repair timeline adapted from Midwood et al. J. Biochem. 2004

IMPACT
ACUTE VS. SUBACUTE



Tissue repair timeline adapted from Midwood et al. J. Biochem. 2004

IMPACT

ACUTE VS. SUBACUTE SUMMARY

Acute (≤ 3 days) exposures for comet can minimize the potentially confounding effects of chronic inflammation and/or tissue repair

Impact

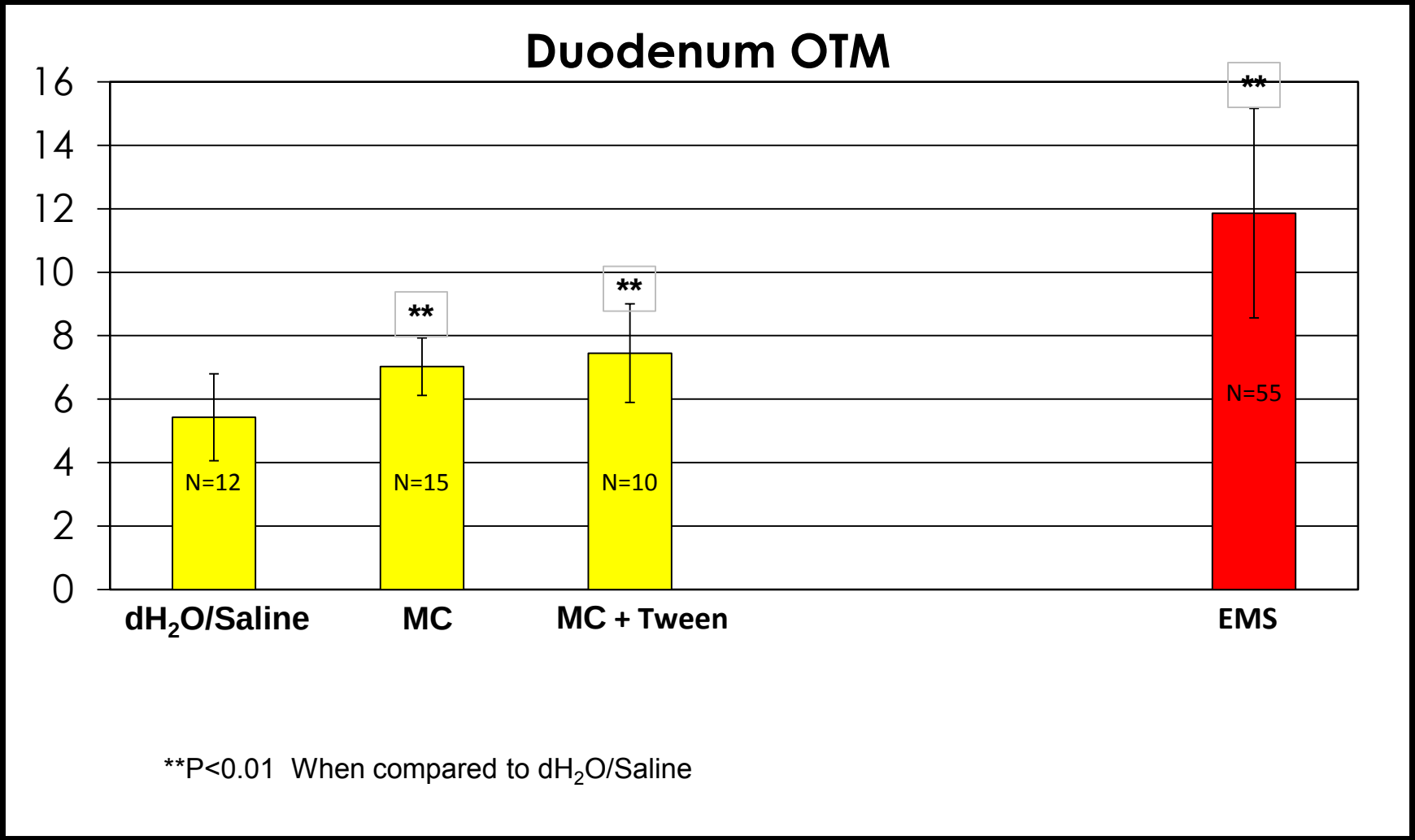
VEHICLE / SITE OF CONTACT

Comet Study design:

Only studies with the exact same following features were included:

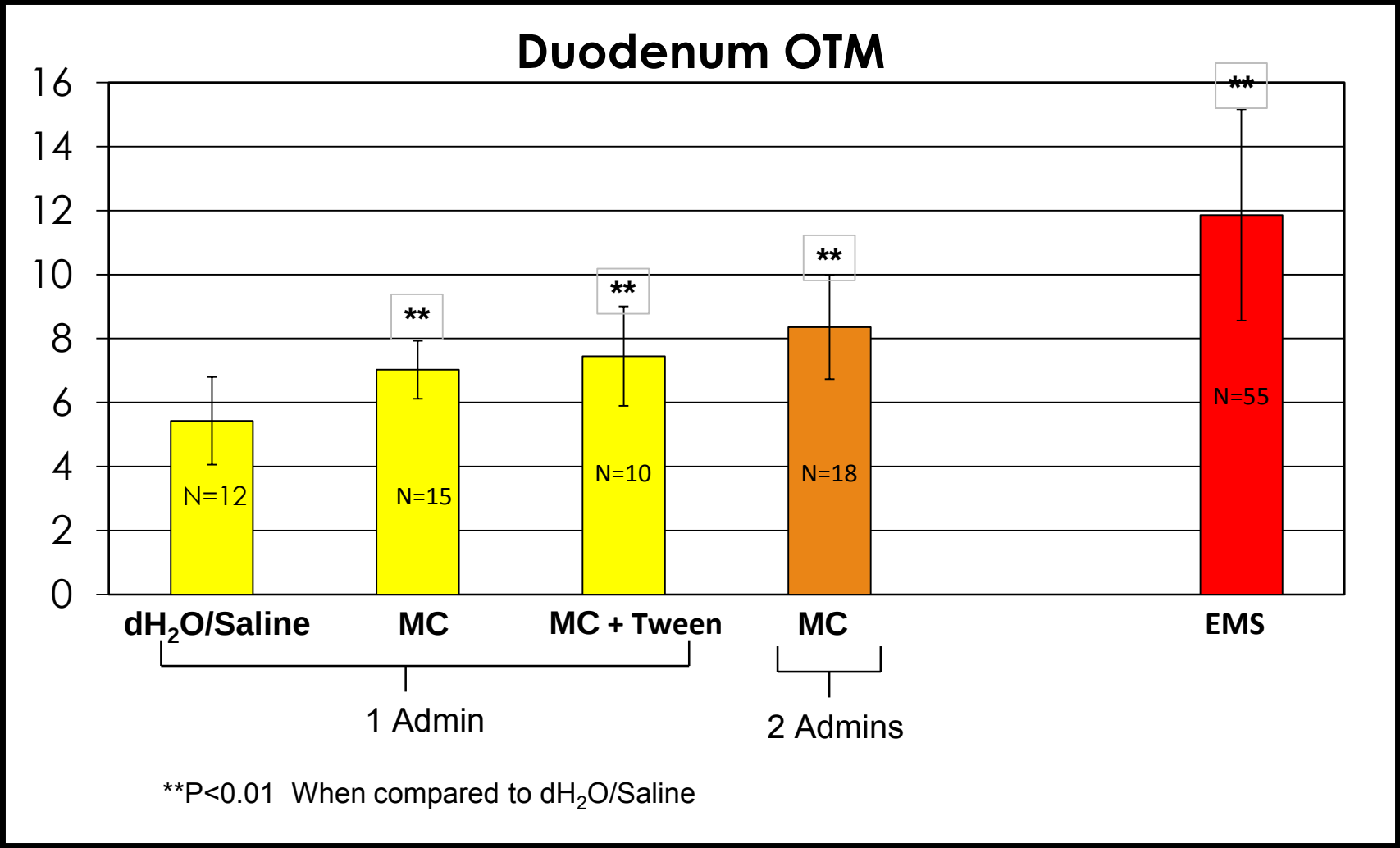
- Vehicle (CAS, composition, viscosity)
- Oral administration
- 1 or 2 dose administrations
- Sprague Dawley Rats
- Liver and Duodenum

IMPACT
VEHICLE / SITE OF CONTACT

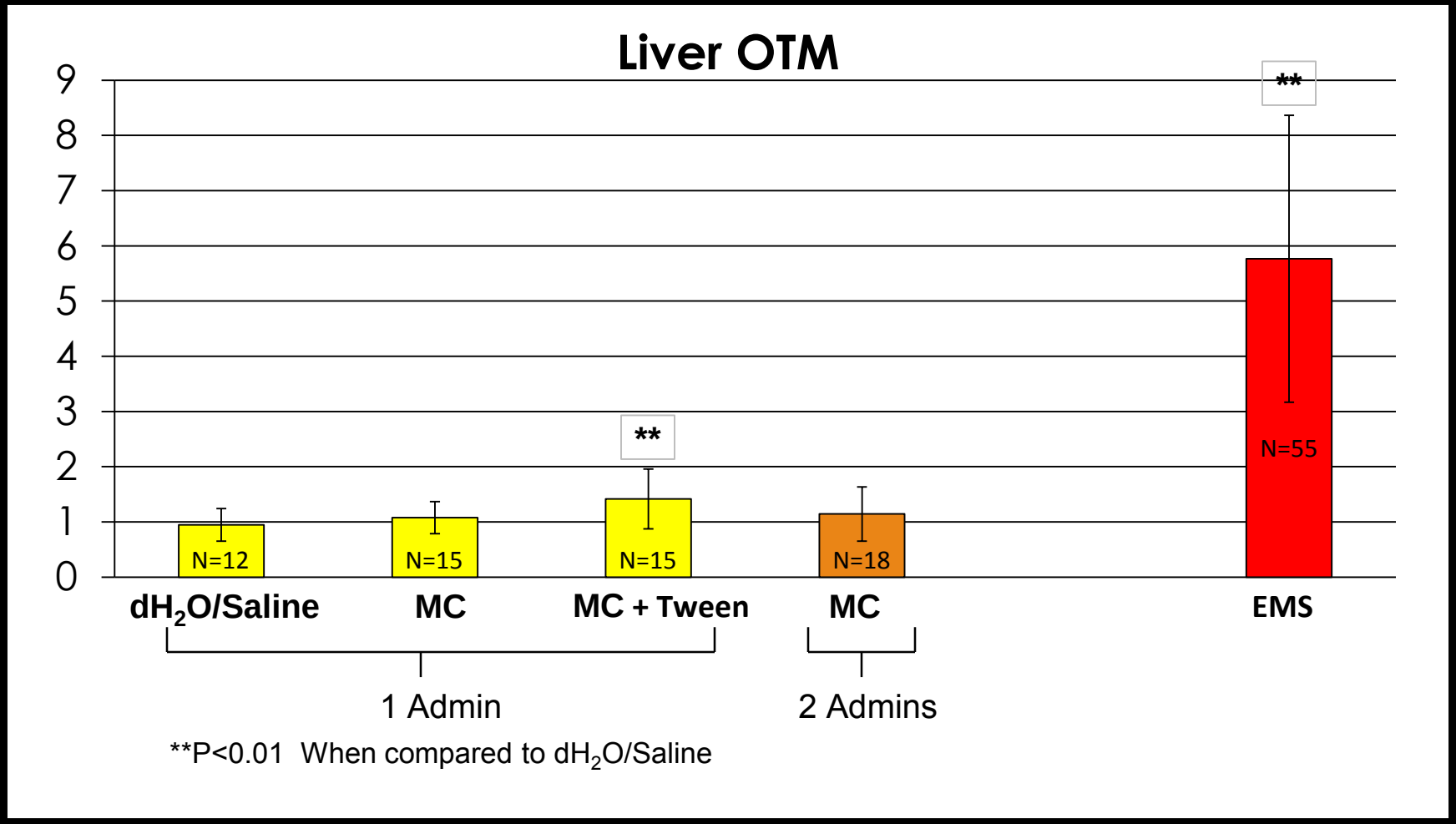


**P<0.01 When compared to dH_2O/Saline

IMPACT
VEHICLE / SITE OF CONTACT



IMPACT
VEHICLE / SITE OF CONTACT



IMPACT

VEHICLE / SITE OF CONTACT SUMMARY

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Some vehicles can influence the DNA migration response, depending on the tissue, route, and number of administrations

IMPACT

TISSUE / CELL SELECTION

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Case Study 5:

- Non-toxic compound in the duodenum

- Pre-Comet study Info:
  - No mortalities up to 2000 mg/kg
  - Dose formulations pH 3

- Study Design:
  - 2 daily oral doses at 0, 250, 500, 1000 mg/kg
  - 5 animals per dose
  - Sample time: 4 hrs

IMPACT

TISSUE / CELL SELECTION

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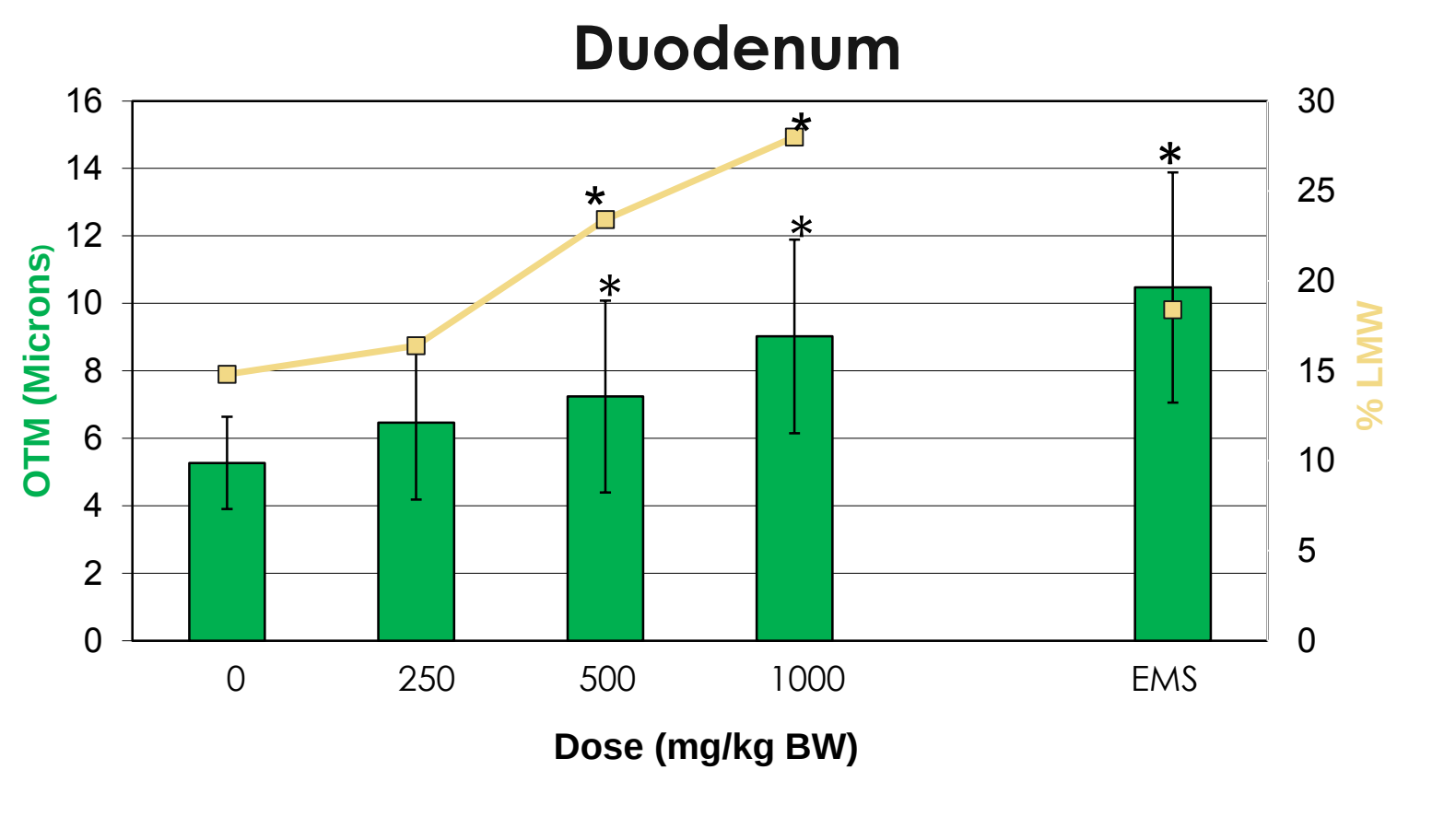
Results:



IMPACT

TISSUE / CELL SELECTION

- Post Study Data:
  - Mild-moderate inflammation at 500 & 1000 mg/kg
  - Increases in %LMW at 500 & 1000 mg/kg
  - Hunched posture/lethargy at 500 &1000 mg/kg

- Additional Data:
  - Negative in Ames*, ML, and mouse MN
  - Positive *in vitro* MN

*Tested *in vivo* due to genotoxicity of similarly structured compound

IMPACT

TISSUE / CELL SELECTION

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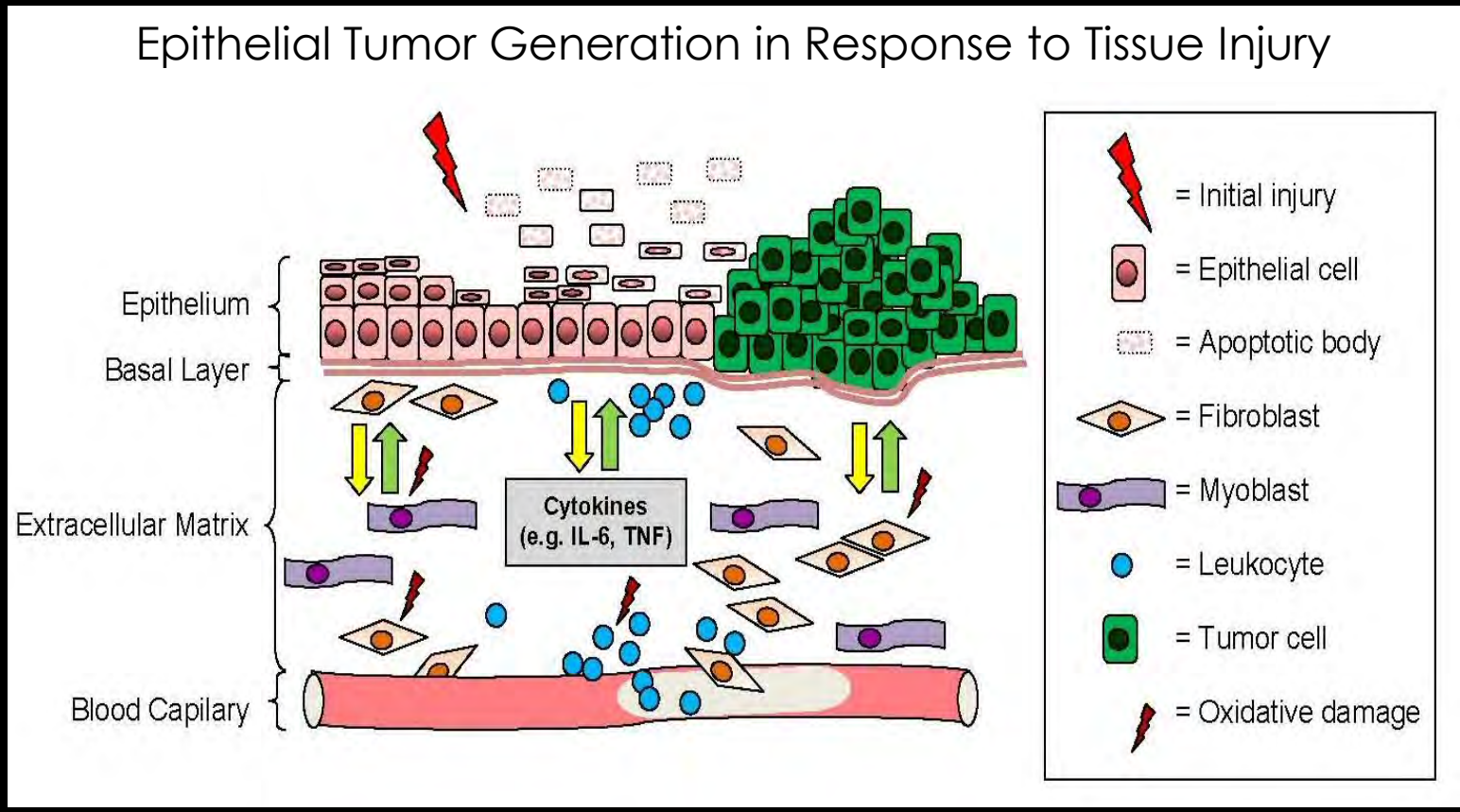
Case Study 5 Conclusions:

- Inflammation, clinical observations and %LMW were indicative of a cytotoxic effect in the duodenum caused by the corrosivity of the acidic (pH 3) doses
- Increase in DNA migration was therefore cytotoxic and not genotoxic
- Comet Result: Negative

IMPACT

TISSUE / CELL SELECTION



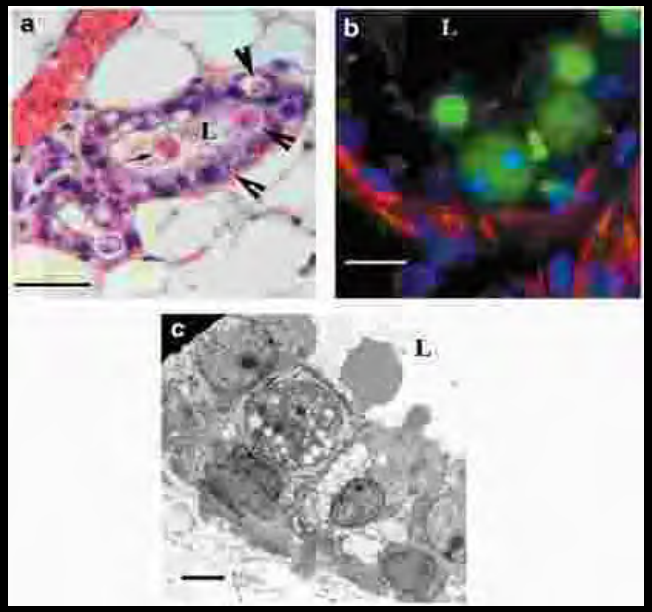
The detection of damage in the progressively dying epithelial cells is less reliable and less indicative of risk than damage in the cells of the ECM

Impact

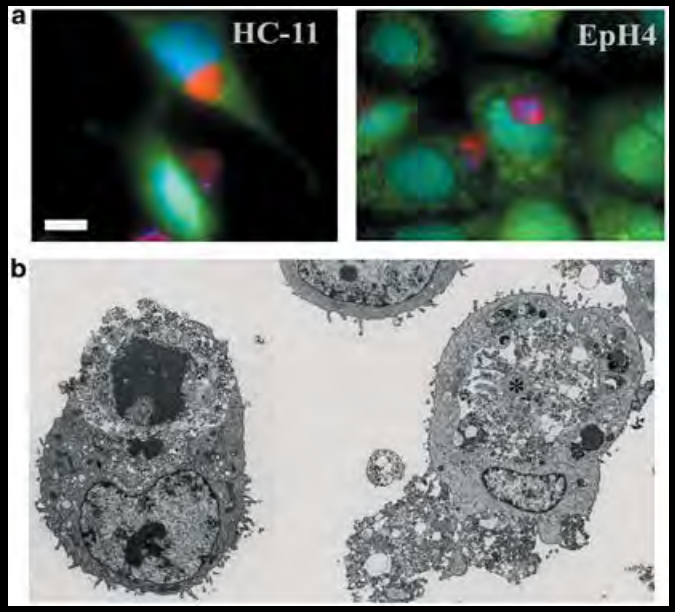
Tissue / Cell Selection

Phagocytosis of apoptotic bodies by neighboring epithelial cells can result in the appearance of non-mutation related micronuclei / DNA fragments in the cytoplasm of undamaged and viable cells

in vivo



in vitro



Monks, D. et al. Cell Death Differ. (2005) 12: 107-114

IMPACT

TISSUE / CELL SELECTION

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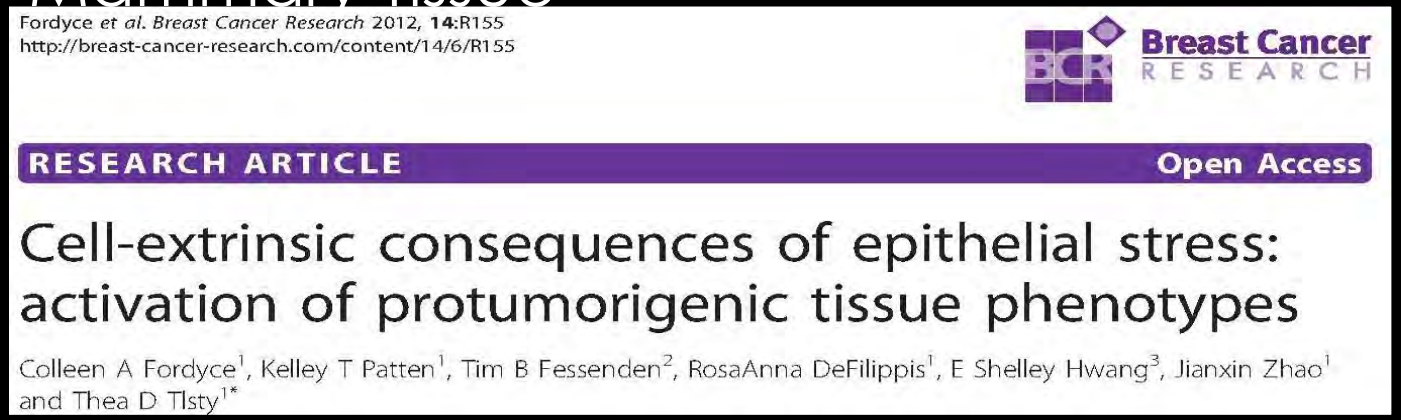
Mammary Tissue



Fordyce *et al. Breast Cancer Research* 2012, **14**:R155
http://breast-cancer-research.com/content/14/6/R155

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

Cell-extrinsic consequences of epithelial stress: activation of protumorigenic tissue phenotypes

Colleen A Fordyce[1], Kelley T Patten[1], Tim B Fessenden[2], RosaAnna DeFilippis[1], E Shelley Hwang[3], Jianxin Zhao[1] and Thea D Tlsty[1*]

Mammary, skin, and bladder

REVIEWS

TUMOUR MICROENVIRONMENT

Fibroblasts in cancer

Raahu Kalluri[*‡§] *and Michael Zeisberg*[*]

392 | MAY 2006 | VOLUME 6

IMPACT

TISSUE / CELL SELECTION

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Forestomach

February 2013 | Volume 9 | Issue 2 | e1003251

PLOS | GENETICS

Inflammation-Mediated Genetic and Epigenetic Alterations Drive Cancer Development in the Neighboring Epithelium upon Stromal Abrogation of TGF-β Signaling

B. R. Achyut[1], David A. Bader[2], Ana I. Robles[3], Darawalee Wangsa[4], Curtis C. Harris[3], Thomas Ried[4], Li Yang[1]*

Forestomach, glandular stomach, esophagus,

Nature. 2004 November 18; 432(7015): 332–337. doi:10.1038/nature03096.

Stromal fibroblasts in cancer initiation and progression

Neil A. Bhowmick[1,3,4], Eric G. Neilson[2,4], and Harold L. Moses[1,2,4,*]

IMPACT

TISSUE / CELL SELECTION

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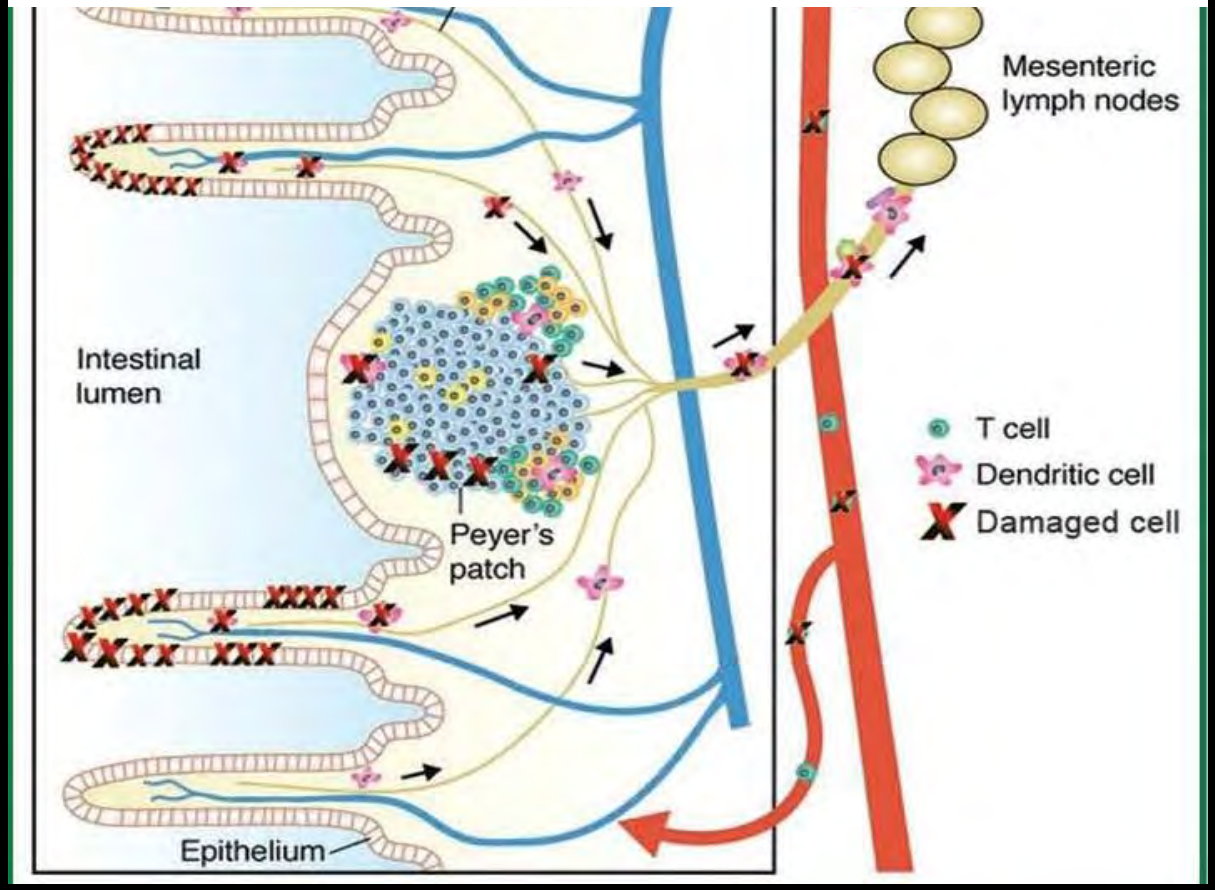
Systemic genotoxicity of intestinal inflammation



Westbrook et.al Cell Cycle 2009 vol. 18 (16)

IMPACT

TISSUE / CELL SELECTION SUMMARY

- Mild to moderate inflammation induced by irritants and/or corrosive substances can increase DNA migration, both locally and systemically

- DNA damage in the ECM cells may be more indicative of the mutagenic potential than damage detected in the progressively dying epithelial cells

CONCLUSIONS

- Cytotoxicity measurements that may be most relevant to comet include:
  - Minimal to moderate inflammation
  - Glycogen depletion (liver only)
  - Hypertrophy/hyperplasia
  - Significant (≥10%) decrease in body weight gain
  - Significant ($p<0.05$) increase in %LMW
  - Increased ALT/AST/GLDH
  - Increased IL-6/TNFa

CONCLUSIONS

- Acute (≤3 days) exposures minimize the effects that can potentially confound comet
- Irritants and/or corrosive substances (including the vehicle) can indirectly induce or enhance DNA migration
- Due to the apoptotic nature of epithelial cells, an indiscriminate collection of cells from the whole tissue should be evaluated with comet