Group	Treatment	No. of Animals to be Sacrificed after Single Dose Administration
		Liver & Bladder
		3-4 hrs
1	Vehicle Control [PEG-400]	6
2	Sample A	6
3	Sample B	6
4	Sample C	6
5	Positive Control [MMS 40 mg/kg]	6

RESULTS

Summary of the Comet Tail Intensity Data of ICR Mice Liver Cells for each Sample Group after 3-4 hrs Treatment with Samples A, Sample B and Sample C

Liver				
Dose	N	% Tail Intensity[1] Mean ± S.D		
		2-8°C	Room Temperature	Expected Results
Vehicle Control [Polyethelene glycol, PEG]	6	0.32 ± 0.07	2.00 ± 1.37	-
Sample A	6	2.00 ± 0.48*	57.75 ± 6.36*	+
Sample B	6	0.45 ± 0.20	3.71 ± 3.28	-
Sample C	6	0.37 ± 0.20	1.76 ± 1.14	-
Positive Control [MMS, 40 mg/kg]	6	5.68 ± 0.45 *	80.45 ± 2.24*	+

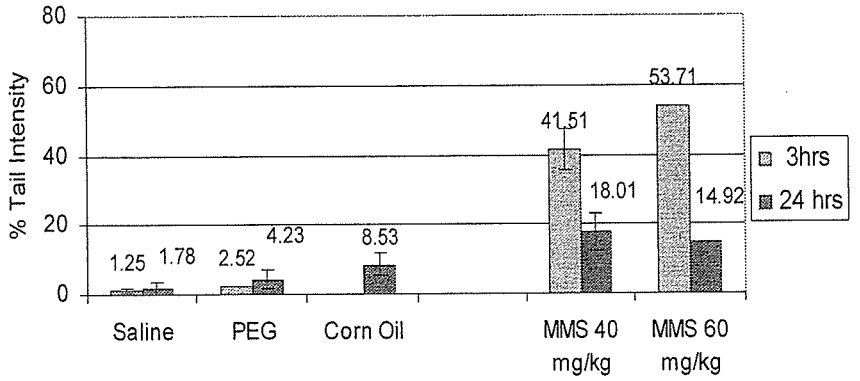
[1] Mean of 6 animals medians
N= Number of animals per group
MMS= Methyl methanesulfonate
* $p < 0.05$, when compared with negative control (t-test)

Summary of the Comet Tail Intensity Data of ICR Mice Bladder Cells for each Sample Group after 3-4 hrs Treatment with Samples A, Sample B and Sample C

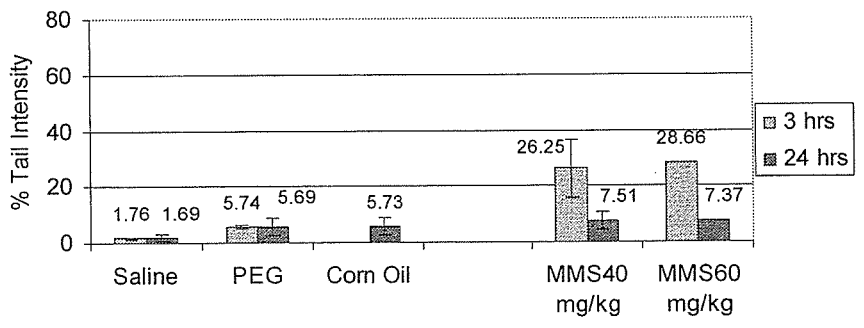
Bladder				
Dose	N	% Tail Intensity[1] Mean ± S.D		
		2-8°C	Room Temperature	Expected Results
Vehicle Control [Polyethelene glycol, PEG]	6	0.78 ± 0.60	7.46 ± 4.47	-
Sample A	6	1.02 ± 0.73	51.37 ± 8.34*	+
Sample B	6	2.24 ± 1.94	15.41 ± 5.25*	+
Sample C	6	1.8 ± 0.39*	16.79 ± 8.92*	+
Positive Control [MMS, 40 mg/kg]	6	1.5 ± 0.53*	69.92 ± 5.87*	+

[1] Mean of 6 animals medians
N= Number of animals per group
MMS= Methyl methanesulfonate
* $p < 0.05$, when compared with negative control (t-test)

DNA Damage in Liver Cells 3 & 24 hrs Post-treatment



DNA Damage in Bladder Cells 3 & 24 hrs Post-treatment



Conclusion

Temperature is an important factor in the sensitivity of the Comet Assay and it needs to be addressed and taken into account for the testing procedures in the international validation studies.

Acknowledgments BioReliance Staff:

Chuong Do, Michael Joseph, Tawney Huston,
Mirna Arevalo, Serita Kendrick, Jen Clair
&
Dr. Buba Krsmanovic

In vitro Comet assay
-A possible candidate as a member of the standard test battery-

Masamitsu Honma

Division of Genetics and Mutagenesis

National Institute of Health Sciences

Genotoxicity Tests (Prediction of Carcinogenicity)

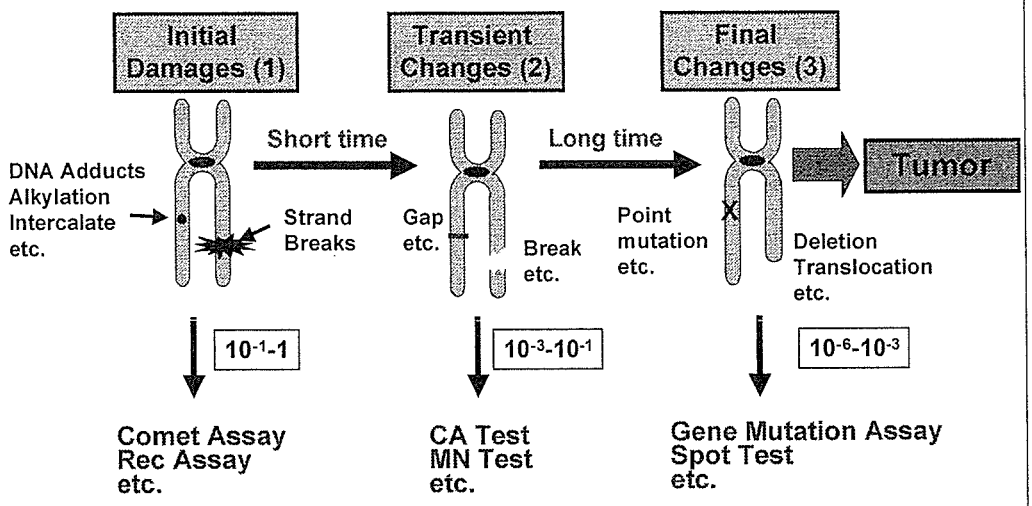
In vitro Tests

1, Rec Assay
2, Phage Induction Test
3, Umu Test
4, Bacterial Reverse Mutation Test (Ames assay)
5, Bacterial Forward Mutation Test
6, Mutation Assay Using *S. Cerevisiae*
7, Mammalian Gene Mutation Assay
8, UDS Test
9, SCE Test
10, Chromosome Aberration (CA) Test
11, Micronuclei (MN) Test
12, Comet Assay
13, Cell Transformation Test
etc.

In vivo Tests

1, Micronuclei Test
2, SCE Test
3, Chromosome aberration Test
4, UDS Test
5, Endogenous Gene Mutation Assay (Hprt, GPA, HLA, etc.)
6, Transgenic Gene Mutation Assay (MutaMouse, BigBlue, etc.)
7, Spot Test
8, Comet Assay
etc.

Genotoxic Endpoints



Standard battery of Genotoxicity Tests in ICH (1997)

In Vitro; 2 of 3 Tests	● Bacterial Reverse Mutation Assay (3) ● Chromosome Aberration Test (2) ● Mouse Lymphoma Assay (3)
In Vivo; 1 test	● Micronuclei Test (2)

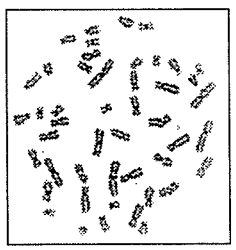
New Concept of *In Vitro* Genotoxicity Test System (MMS Collaborative Study)

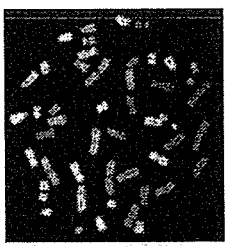
1. Use of human cell and human metabolic system
   -biological relevance for human hazard identification
2. Reasonable tests battery
   -consisting of category 1, 2, and 3 tests for screening wide variety of genotoxicity
3. Sequential analysis in a single treatment
   -elucidation of genotoxic mechanisms

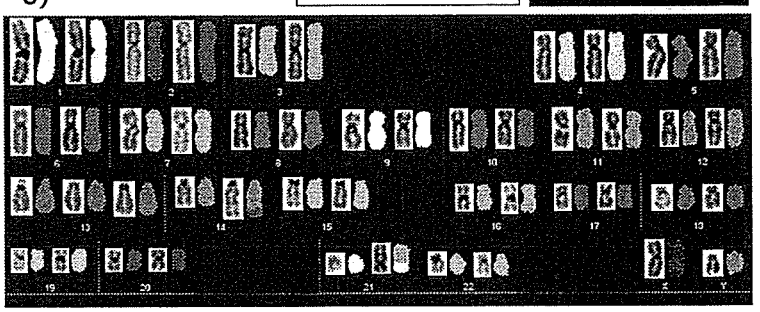
TK6 cell

TK6 (ATCC No. CRL-8015)
(Human lymphoblastoid cell line)

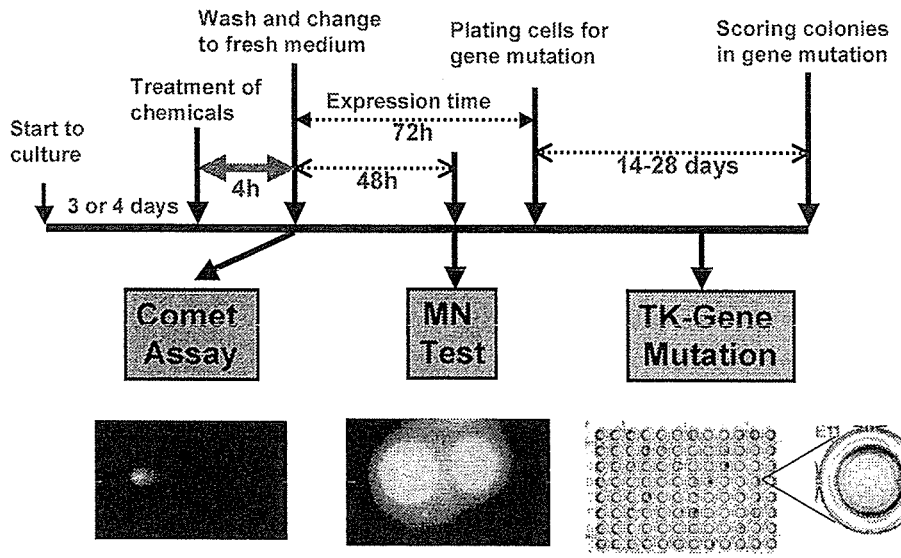
47, XY, 13+,
t(14; 20), t(21; 3)







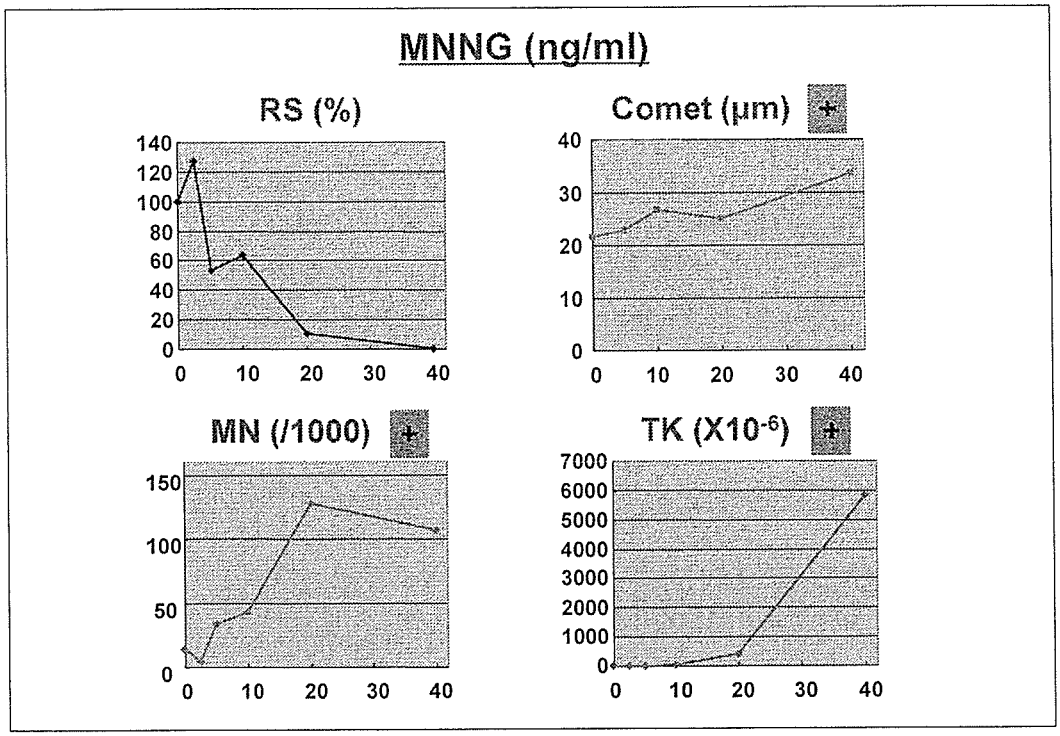
Test Battery and Treatment

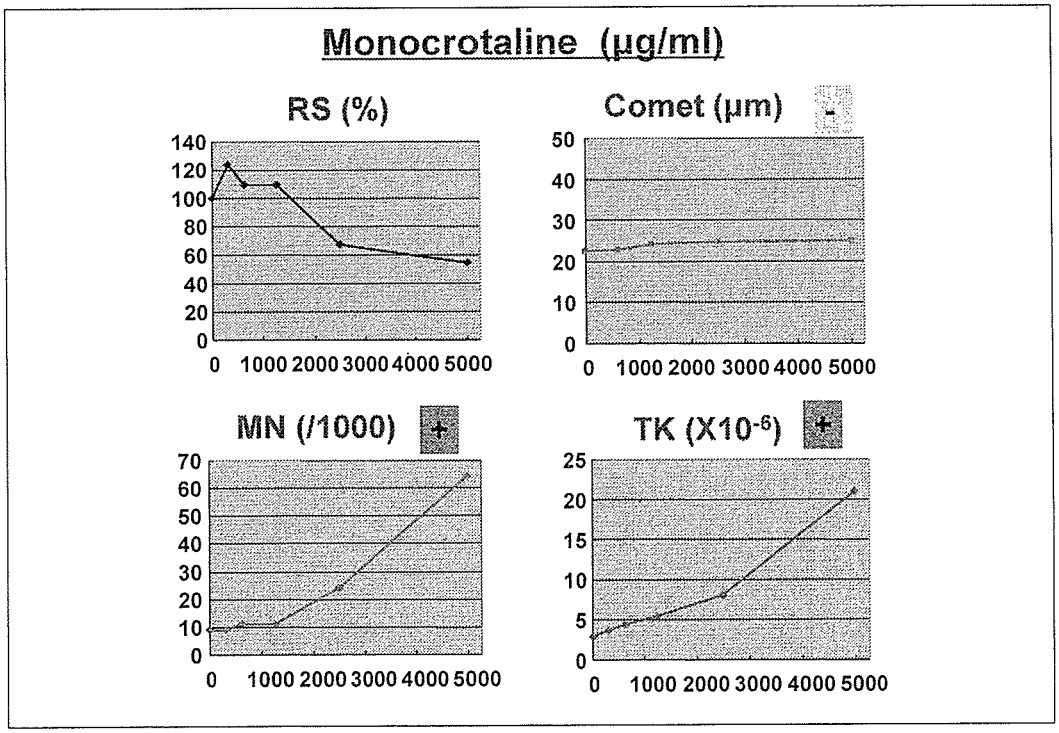


Test Chemicals

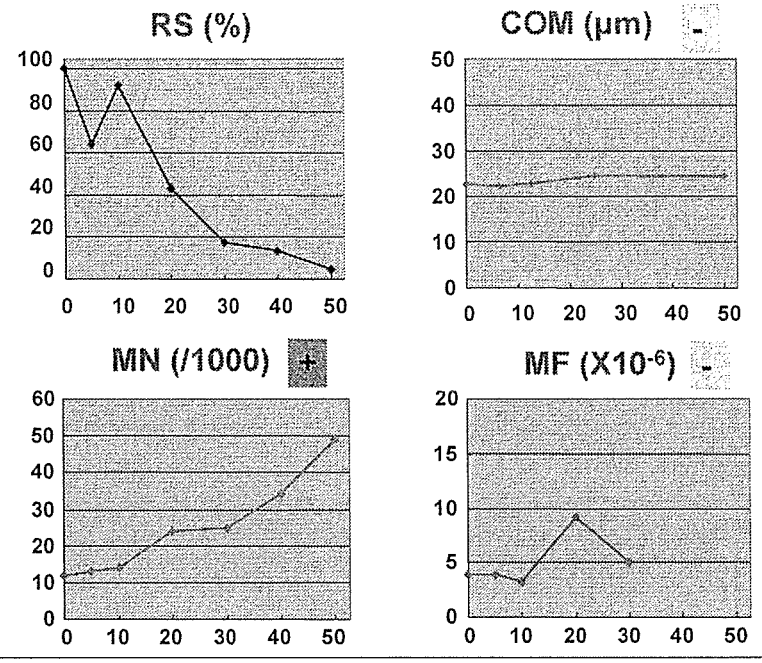
	Chemicals	Ames	CA	MLA
1	Acrylamide			
2	N-Aminoethyl ethanolamine			
3	Bleomycin sulfate			
4	Camptothecin			
5	Catechin			
6	Colchicine			
7	Cytocine arabinoside			
8	5-Fluorouracil			
9	Glycidamide			
10	Griseofulvin			
11	Hexamethyl phosphoramide			
12	Hydroxyurea			
13	Methotorexate			
14	MNNG			
15	Monocrotaline			
16	4NQO			
17	Quercetin			
18	Vinblastine sulfate			

Negative Positive Weak positive

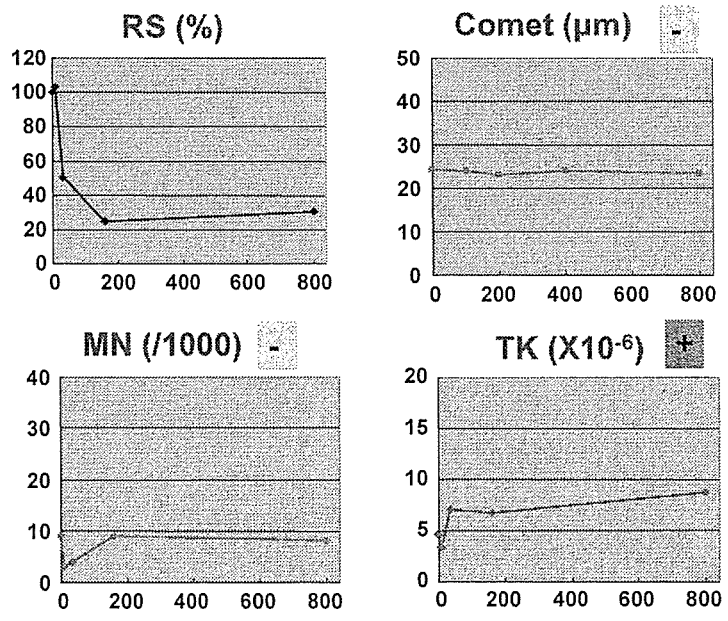

MNNG (ng/ml)
RS (%)
Comet (μm)
+
MN (/1000)
+
TK (X10-6)
+


Monocrotaline (μg/ml)
RS (%)
Comet (μm)
-
MN (/1000)
+
TK (X10-6)
+

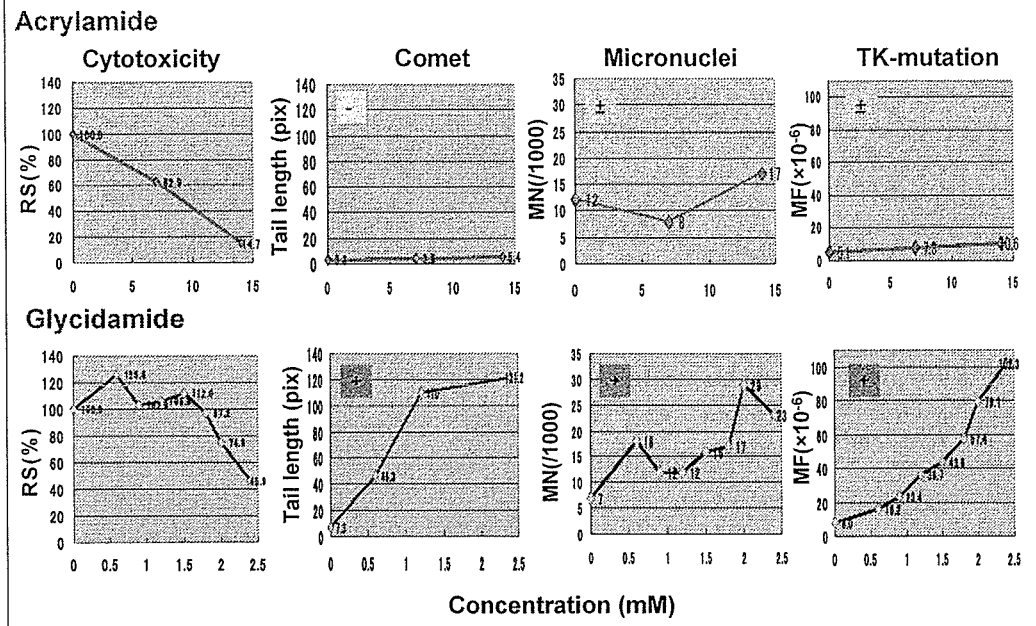
Colchicine (ng/ml)



Hydroxyurea (µg/ml)



Acrylamide and Glycidamide



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Mutation Research 603 (2006) 151–158

Genetic Toxicology and Environmental Mutagenesis

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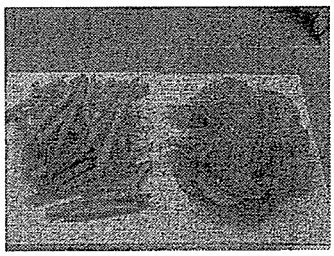
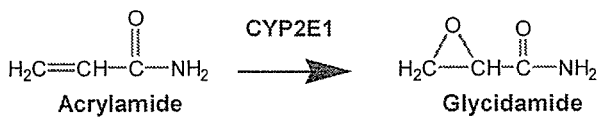
Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

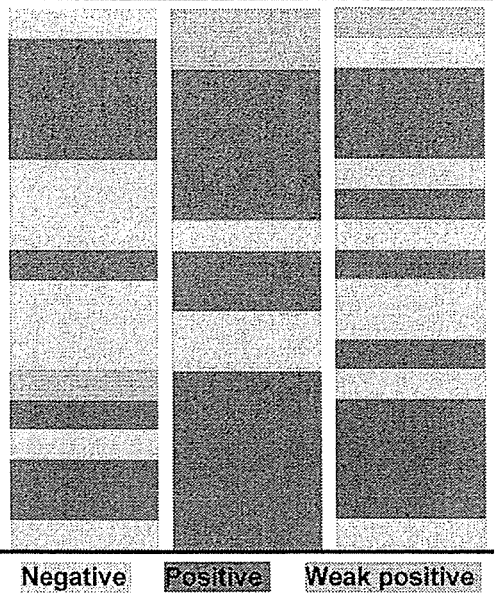
Naoki Koyama[a,b,c], Hiroko Sakamoto[a], Mayumi Sakuraba[a], Tomoko Koizumi[a], Yoshio Takashima[a], Makoto Hayashi[a], Hiroshi Matsufuji[b], Kazuo Yamagata[b], Shuichi Masuda[c], Naohide Kinae[c], Masamitsu Honma[a,*]





Test Results

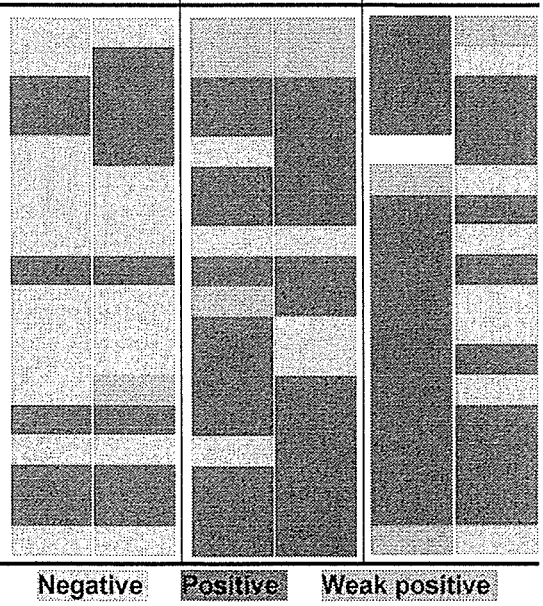
	Chemicals	Comet	MN	TK
1	Acrylamide			
2	N-Aminoethyl ethanolamine			
3	Bleomycin sulfate			
4	Camptothecin			
5	Catechin			
6	Colchicine			
7	Cytocine arabinoside			
8	5-Fluorouracil			
9	Glycidamide			
10	Griseofulvin			
11	Hexamethyl phosphoramide			
12	Hydroxyurea			
13	Methotorexate			
14	MNNG			
15	Monocrotaline			
16	4NQO			
17	Quercetin			
18	Vinblastine sulfate			



Negative Positive Weak positive

Comparison of Test Results

	Chemicals	Ames	Comet	CA	MN	MLA	TK
1	Acrylamide						
2	N-Aminoethyl ethanolamine						
3	Bleomycin sulfate						
4	Camptothecin						
5	Catechin						
6	Colchicine						
7	Cytocine arabinoside						
8	5-Fluorouracil						
9	Glycidamide						
10	Griseofulvin						
11	Hexamethyl phosphoramide						
12	Hydroxyurea						
13	Methotorexate						
14	MNNG						
15	Monocrotaline						
16	4NQO						
17	Quercetin						
18	Vinblastine sulfate						



Negative Positive Weak positive

Consistency of Results between Comet Assay and Other Existing Tests

Comet vs Ames

		Comet +9	Comet - 9
Ames	+ 6	6	0
	- 12	3	9

Const. : 83%

Comet vs CA

		Comet +9	Comet - 9
CA	+ 15	8	7
	- 3	1	2

Const. : 56%

Comet vs MLA

		Comet +9	Comet - 9
MLA	+ 17	8	9
	- 0	1	0

Const. : 47%

Consistency between Genotoxic Test Results

Category II

Comet vs Ames

		Comet +9	Comet - 9
Ames	+ 6	6	0
	- 12	3	9

Const. : 83%

CA vs MN

		MN +15	MN - 3
CA	+ 15	13	2
	- 3	2	1

Const. : 78%

Category III

MLA vs TK

		TK +11	TK - 6
MLA	+ 17	11	6
	- 0	0	0

Const. : 65%

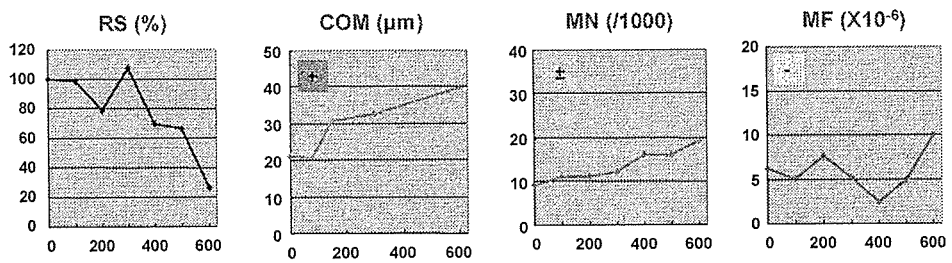
Comet Positive, but Ames Negative Chemicals

Comet vs Ames

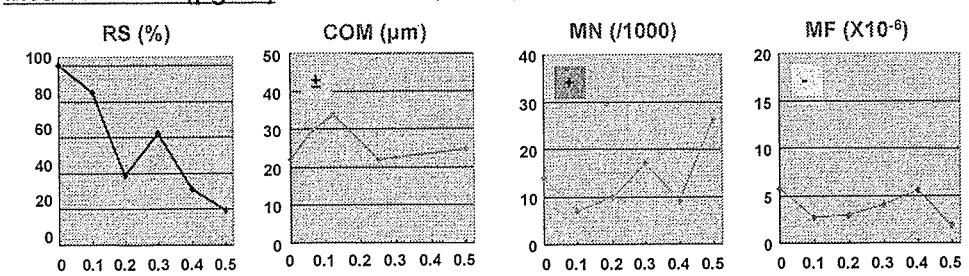
		Comet	
		+9	- 9
Ames	+ 6	6	0
	- 12	3	9

N-Aminoethyl ethanolamine	Aneugen?, MLA positive
Methotorexate	MLA positive, V79-Hprt negative
Catechin	Antimutagen?

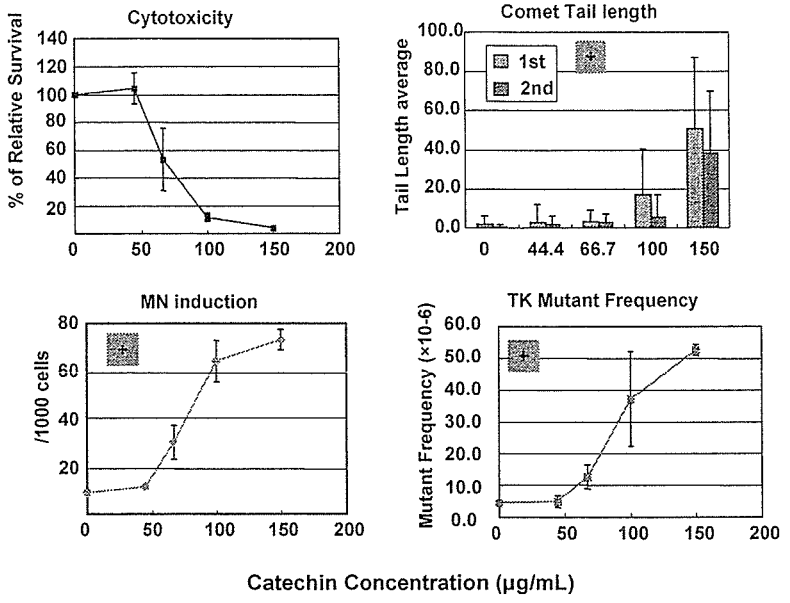
Aminoethyl ethanolamine (µg/ml) Ames: - , CA: ±, MLA: +



Methtorexate (µg/ml) Ames: - , CA: +, MLA: +



Genotoxicity of Catechin



Summary

- The in vitro Comet assay can detect Ames positive chemicals at high probability.
- The in vitro Comet assay can not detect indirect DNA acting mutagens including spindle poisons and metabolic antagonists.
- Some chemicals show positive in the Comet assay, but not in the Ames assay. Their characterization should be analyzed.
- Theoretically as well as practically, the in vitro Comet assay could be replaced to the Ames assay in the genotoxic battery test.
- We propose the new concept in vitro genotoxic test battery.

1. Use of human cell and human metabolic system
2. Reasonable tests battery
3. Sequential analysis in a single treatment

Case Study 2 – Sensitivity of the alkaline Comet Assay to buffer temperature during unwinding and electrophoresis

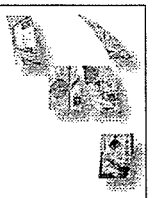
Escobar Patricia, Do Chuong and Joseph Michael
Genetic Toxicology Department
BioReliance, Invitrogen BioServices

The Comet Assay, also known as Single Cell Gel Electrophoresis (SCGE), has the ability to detect DNA damage at a single cell level. This assay is increasingly being used in genotoxicity testing. Recommendations for appropriate performance of the test have been published but, as part of the validation initiative, the protocol of the Comet assay has to be established and all the technical issues need to be taken into account.

As previously discussed by Speit et al. (1999) temperature is a technical variable that can affect the sensitivity and resolving power of the Comet Assay. In experiments conducted at BioReliance temperature sensitivity was found to be very important in detecting compounds that are weak positives.

The case study presented here is one example of an in vivo Comet assay and how the temperature affected the overall results in calling the compound positive. In this validation study mice were treated with 3 unknown compounds: Sample A, Sample B, Sample C. There were also vehicle (PEG), and a positive (MMS 40 mg/kg) control groups. The animals were exposed for 3 hours with the 3 unknown compounds before the liver and bladder were removed. A cell suspension was obtained by mincing the liver and scraping the internal lining of the bladder. From the cell suspension 4 comet slides were prepared per animal and slides remained in the lysis solution for at least 24 hours. Unwinding was for 20 minutes and electrophoresis was for 30 minutes at 0.7 V/cm, for all the experiments. One set of slides were run in the refrigerator (actual buffer temperature 7.0 ± 0.5 °C) and the other set were run at room temperature (actual buffer temperature 21 ± 1 °C). The expected results were observed in the room temperature data but not in the refrigerated data. Actual data will be presented at the symposium.

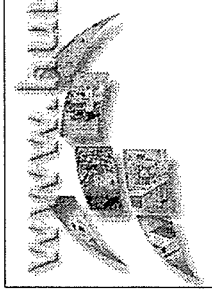
Temperature is an important factor in the sensitivity of the Comet Assay and it needs to be addressed and taken into account for the testing procedures in the international validation studies.



Limitations of the Comet Assay -1

The Pros and Cons of the Comet Assay in Human Risk Assessment

Sapporo, August 2006

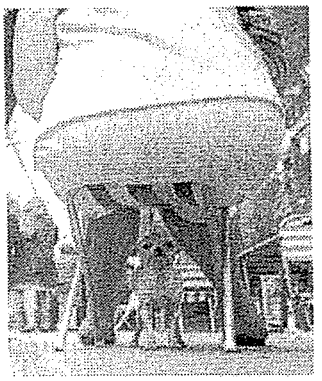


Content

- Risk assessment
  - What are we really trying to do?
- The assay
  - Is it a tool that will help us to do it?
- The data
  - Is it believable?
- The regulators
  - Do they believe it?

Risk Assessment

- Identify a hazard
- Assess the risk associated with the hazard
- Assess the consequences (to man)



Probability: High
Severity: Serious

Is there a place for the Comet assay in the current test battery?

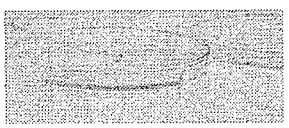
- Battery is there to identify hazard
- In vitro – do we need another assay?
  - General consensus is probably not
- In vivo – do we need another assay
  - General consensus is probably yes

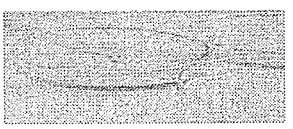
Sobels' Parallelogram

Rodent

The comet assay
Can be used in any
of these positions

Human





Is the data believable?

- Already some data in the literature which has caused some concern (rodent)
- Strenuous exercise can cause comets (oxidative damage) in leukocytes in man.
- What impact does toxicity have on comet formation?

However.....

- Much more published data where the comet assay has been critical in deciding the eventual fate of both drugs and industrial materials. (Review in Mut. 20 (4))

Do regulators believe in the data?

- Following real examples provided by Andreas Hartmann, Novartis

4