the presence of S9-mix, although it is known that these chemicals except for 2AA are not metabolically activated.

1. Ethymethansulfonate (EMS): alkylating agent
2. Mitomycine C (MMC): cross-linker
3. 2-Aminoanthracene (2AA): aromatic hydrocarbon
4. Cycloheximide (CHX): inhibitor for protein synthesis
5. Triton-X (TRX): detergent

Note) VMT purchases these chemicals together, and deliver them to each laboratory.

2-7. Top concentration, dose selection, and solvent

The laboratory should conduct the Comet assay for the chemicals by the appointed concentrations and solvent as follows. Each experiment consists of one solvent control and 6 concentration of the test chemical. Two-fold serial dilutions are prepared from the top concentration.

1. EMS: DMSO
   S9-mix +/-; 0, 62.5, 125, 250, 500, 1000, 2000 ug/ml
2. MMC: physiological saline
   S9-mix +/-; 0, 0.125, 0.25, 0.5, 1, 2, 4 ug/ml
3. 2AA: DMSO
   S9-mix -; 0, 125, 250, 500, 1000, 2000, 4000 ug/ml
   S9-mix +; 0, 0.0625, 0.125, 0.25, 0.5, 1, 2 ug/ml
4. CHX: ethanol
   S9-mix +/-; 0, 62.5, 125, 250, 500, 1000, 2000 ug/ml
5. TRX: physiological saline
   S9-mix +/-; 0, 6.25, 12.5, 25, 50, 100, 200 ug/ml

For EMS, MMC, 2AA, these concentrations cover concentrations showing enough cytotoxicity and positive responses in the TK-gene mutation assay in TK6 cells (Honma et al., 1994 and unpublished data). The top concentrations are more than 10-folds of those in the TK-mutation assay. CHX did not show any cytotoxicity and Comet-response in TK6 cells even at the highest concentrations (5000 ug/ml) (Henderson et al., 1998). Although the in vitro cytotoxicity of TRX in TK6 cells are not clear, it exhibits clear cytotoxicity less than at 200 uM (50 ug/ml) in A549 cells (Vock et al., 1998). We don't take positive control even for experiments with non-genotoxic chemicals.

2-8. Evaluation of cytotoxicity

The possible cytotoxicity tests for in vitro Comet assay are indicated below. In the pre-validation study, 1) and 2) must be taken for evaluating the cytotoxicity and comparing their characteristics. The laboratory must conduct both 1) and 2) with the Comet assay at the same time. Optionally, other tests could be taken if the laboratory prefers.

1) Trypan blue dye exclusion test just after the treatment
2) Relative cell growth for 24 h after the treatment
3) Trypan blue dye exclusion at 24 h later after the treatment
4) Relative survival (colony formation) just after the treatment
5) Neutral diffusion assay just after the treatment
6) Dual dye viability staining just after the treatment (Strauss et al., 1991)
7) Others (ATP concentration, mitotic index, etc.)

2-9. Standard procedure of treatment for TK6

i) The laboratory thaws the stoked cells into 50 ml of culture medium in a TS-75 culture bottle and starts cell culture. After several days, logarithmic growing TK6 cells are prepared approximately at 2 X 10^5 cells/ml with culture medium and divided into 15 ml plastic tubes by 9.9 ml aliquots for an experiment without S9-mix. When S9-mix is used, 9.4 ml cell suspensions and 0.5 ml S9-mix are placed into the tubes.

ii) The 0.1 ml of serially diluted chemical is added into the tube for starting treatment. The tube is closed tightly and incubated at 37C with gentle shaking on a rocker platform in an incubator for 4 hours.

iii) After the treatment, 1 ml of culture is taken into a new tube and is centrifuged at low speed (approximately 1,000 r.p.m. for 5 min), and supernatant is discarded. Each culture is washed with 1 ml of cold HBSS solution with 20mM EDTA and 10% DMSO, once by re-suspension and centrifugation. The cells are then re-suspended in 0.5 ml of the cold HBSS solution again. The cell suspensions are used for Comet slide preparation.

iv) After the treatment, a small portion of cell cultures are taken and examined for trypan blue dye exclusion test.

v) The remained culture is also centrifuged at low speed (approximately 1,000 r.p.m. for 5 min), and supernatant is discarded. Each culture is washed with 5 ml of fresh medium once by re-suspension and

centrifugation. The cells are then re-suspended in 10 ml of fresh medium and transferred to culture bottles (TS-25) or culture dishes. The cell density is measured by a hemocytometer or an automatic cell counter before starting culture. The cultures are incubated at 37C in a humidified incubator gassed with 5% CO_2 and in air. Twenty-four hours later, the cell density is measured again. The relative cell growth to the solvent control is calculated.

3. SLIDE PREPARATION

After the treatment, the cells are washed and made as single cell suspension with or without typsinazation. The cell sample for Comet assay should be finally suspended in cold HB solution with 20mM EDTA and 10% DMSO. The cell suspension sample and 0.5 % low melting agarose are mixed by 1:9 for preparing Comet slides.

4. STATISTICS

We do not have any appropriate statistical analysis in in vitro Comet assay. The results of the pre-validation study may be helpful to establish the statistics.

E. DATA AND REPORTING

The information of data, reporting, and tabular form will be announced later.

F. ARCHIVES AND REVIEW

The study reports and all of data should be retained according to the SOP in each laboratory. They will be provided to VMT for reviewing.

G. REFERENCES

Henderson, L., et al., The ability of the Comet assay to discriminate between genotoxins and cytotoxins. Mutagenesis, 13, 89-94 (1998)

Honma et al., Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells. Mutation Research 374, 89-98 (1997)

Honma, M., Hayashi M., Shimada H., Tanaka N., Wakuri S., Awogi T.,

Yamamoto K.I, Kodani N.-U., Nishi Y., Nakadate M. and Sofuni T. Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the in vitro chromosomal aberration test. Mutagenesis, 14, 5-22 (1999).

Strauss, GHS., Non-random cell killing in cytopreservation: Implication for performance of the battery of leukocyte tests (BLT), I. Toxic and immunotoxic effects. Mutation Research, 252, 1-15 (1991)

Vock, EH., et al., Discrimination between genotoxicty and cytotoxicity in the DNA double-strand breaks in cells treated with etoposide, melpharan, cisplatin, potassium cyanide, Triton X-100, and gumma-irradiation. Mutation Research, 413, 83-94 (1998)

Draft Minutes

The first meeting for

international *in vitro* Comet assay validation study

Date: August 24, 2007, 3:30-5:30 p.m.

Venue: Hotel East 21,
Tokyo, Japan,

Participants:
M. Hayashi (NIHS), M. Honma (NIHS), L. Schectman (Consultant), R. Tice (NIEHS), Y. Uno (Mitsubishi Pharma), N. Asano (Nitto Denko: MMS president), T. Morita (NIHS), N. Nakashima (PMDA), T. Omori (Kyoto Univ.), P. Clay ( ), P. Escobar (BioReliance), M. Nakajima (Anpyo-Center), M. Ueda (Anpyo-Center), K. Yamakage (FDSC), Y. Nakagawa (FDSC), Y. R. Seo (Kyung Hee Univ.)

Discussion:

1. *In vitro* Comet assay protocol

   When reviewing published or available protocols, they seem overall comparable. A protocol for the international *in vitro* Comet assay validation studies will be basically same as that for *in vivo* Comet assay except for some specific points *in vitro*, e.g. cell preparation method.

2. Low sensitivity of *in vitro* Comet assay

   When reviewing *in vitro* Comet assay data, the assay seems less sensitive compared to the TK mutation assay and the MN assay, as extremely high doses are required to obtain positive results. But it may not be a major issue, because the sensitivity would depend on many factors such as cell types used, experimental conditions, and how to investigate cytotoxicity. This point should be discussed further more.

3. Purpose of validation study

To optimize the study protocol in the pre-validation phase. Finally to investigate linkage to *in vivo* Comet assay results (biological relevance). Need to examine the same chemicals investigating in *in vivo* Comet assay validation (and more chemicals should be examined *in vitro*). Test chemicals should be selected from following four categories: genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens, and non-genotoxic non-carcinogens. After confirmation of robustness of the study protocol and usefulness of the assay (replacement of CA assay and/or MLA assay?), we should work on OECD to establish the guidance for regulatory use. Need to be clarified how to use this assay in regulatory aspects.

4. Items in study protocol

4-1. Cell type:

Primarily, TK6 cells.

Secondarily, mouse lymphoma cells, human lymphocytes, CHL cells, etc.

Comments: Comparison among cell types should be necessary.

Immortal cell lines having normal p53 status would be preferable.

Adhering cell lines vs. suspended cell lines, which is better?

TK6 cell line: easy to induce apoptosis (cf. WTK1: p53 mutant).

Exposure duration, dose range, S9 exposure: sort term exposure does not matter for necrosis/apoptosis.

When and how should cyototoxicity be evaluated? After 24 hr exposure or soon after 3-4 hr exposure?

How many cell lines should be examined? It may depend on numbers of chemicals examined.

An idea is to examine same chemicals with two (or more) cell lines in two (or more) different study groups.

It may be needed to hear worldwide scientist's opinions, which cell lines are preferable for Comet assays.

In pre-validation phase, use of one cell line may be acceptable.

Cell lines used should be from same resource and same passage number.

4-2. Test chemical selection:

Need to be examined with/without S9-mix conditions.

Use of fewer chemicals would be sufficient for optimization of the study protocol.

Following five chemicals will be examined (chemical names will not be coded)

EMS : same as *in vivo* positive control; without S9-mix

MMC : cross-linker; expecting a negative result; without S9-mix

Cycloheximide : non-genotoxic and cytotoxic agent; without S9-mix

Triton X : non-genotoxic and cytotoxic agent; without S9-mix

2AA: with S9-mix

4-3. Treatment time: 4 hrs

4-4. Cytotoxic parameter

When and how should cytotoxicty be checked?

Dye exclusion test with trypan blue: possible to examine immediately after exposure period. This is 1st choice in the pre-validation study (although sensitivity seems bad).

Relative cell growth: 24 hrs after the treatment. 2nd choice.

Relative survival: immediately after exposure period. 2nd choice.

Neutral diffusion assay: immediately after exposure period. 2nd choice.

4-5. Number of samples: duplicate cultures/dose will be used.

4-6. Dose level: at least 5 doses including negative control will be observed.

4-7. Lysis and electrophoresis: basically same as *in vivo* Comet assay conditions.

Room temp in electrophoresis, OK? Need to be examined.

4-8. Statistics: to be discussed.

5. When will this pre-validation study start?

To be discussed.

6. Leading labs selection

To be discussed.

Many EU companies are interested in *in vitro* Comet assay, and thus they will hope to join.

(Leading labs in *in vivo* Comet validation may also be leading labs in *in vitro* Comet validation)

Draft Minutes

The 3rd meeting for

Validation Management Team on Comet Assay

Date: August 26, 2007, 9:00 a.m.-2:30 p.m.

Venue: Hotel East 21,
Tokyo, Japan,

Participants:
M. Hayashi (NIHS), T. Hartung (ECVAM), L. Schechtman (Consultant), R. Tice (NIEHS), Y. Uno (Mitsubishi Pharma), H. Kojima (JaCVAM, NIHS), N. Asano (Nitto Denko: MMS president), R. Corvi (ECVAM), P. Escobar (BioReliance), M. Wind (CPSC), A. Rispin (EPA), T. Morita (NIHS), Y. Nakagawa (FDSC), M. Nakajima (Anpyo-Center), T. Omori (Kyoto Univ.), S.N. Park (KFDA), Y.F. Sasaki (Hachinohe Tech. Coll.), M. Ueda (Anpyo-Center), K. Yamakage (FDSC), S. Tonomori (LSG)

Discussion:

1. Statistical analysis plan for second *in vivo* pre-validation study
   Firstly, Dr. Omori explained how to use EXCEL data sheets provided for the second *in vivo* comet assay pre-validation. There are 3 sets of input columns for each animal, but to input data into 2 of 3 columns is acceptable. Anyone should not change any format provided. Total 3 chemicals were examined in the second *in vivo* comet assay pre-validation. One of them, VMT directed the all dose levels, but other 2 chemicals should be found the optimal dose levels in each laboratory. The laboratory should select dose levels by their way referring to the protocol version 12, section 1.4.1. To share the information how to select the dose levels, each laboratory will report the reason why he/she chooses the top dose. Such information would be helpful to determine the criteria for the dose selection in the main study, and Dr. Escobar will provide a reporting format (tabular format?).
   Secondly, Dr. Omori explained a statistical analysis plan for the second *in vivo* comet assay pre-validation. The objectives of main validation studies are to investigate reproducibility,

and robustness. The group agreed that the estimate (unit) is an animal but not a cell. Primary measures are mean untransformed % tail DNA in the liver of each animal and that in the glandular stomach of each animal. Secondary measures will be also reported, i.e. mean untransformed tail length, mean untransformed Olive tail moment, and median untransformed % tail DNA. Data analysis will be done on assay sensitivity, intra-laboratory variability, comparisons between the dose groups and the vehicle group, inter-laboratory variability, correlation analysis for parts, investigation on the reduction in the number of animals, and using three endpoints for judgment. Regarding Object 2, acceptable criteria on a range of variation of effect will be discussed in the next meeting. Pair-wise tests may produce false positive results, and multiplicity should be also considered for the comparison between the dose groups and the vehicle group. As the 2 chemicals not directed the top dose by VMT may be applied as different dose levels among laboratories, the dose effect should be considered for the slope analysis on estimate. Regarding judgment, there were many opinions as follows: 20% increase of mean % DNA may define positive (cut-off value); each laboratory reports the own call on the positive/negative judgment (put new space for the laboratory call); cut-off value + statistical confidential interval; we will fix the cut-off point later; biological significance *vs* statistical significance; and sensitivity and specificity will be discussed based on the actual data.

2. Member of the VMT

We welcomed Dr. M. Honma as an additional member of VMT under the condition that he and his laboratory do not attend at the validation study (VMT issue). Dr. T. Hartung was replaced by Dr. R. Corvi. Then new members of VMT for the comet assay are Drs. R. Colvi, M. Hayashi, M. Honma, L. Schechtman, R.R. Tice, and Y. Uno. The group also agreed that Dr. R. Tice represents NICEATM and also ICCVAM.

3. Progress, schedule and plan of *in vivo* validation study

Dr. Uno explained progress of the 2nd pre-validation study and the draft schedule and plan for the main validation studies.

Regarding recruitment of main validation studies, 5 laboratories have already applied to join the project. We need to discuss following issues: about the exception for participants (VMT issue); how many laboratories should be participated in the main studies; how many chemicals should be examined in the main studies (20 may not be enough to make final decision); and how many chemicals/laboratory = is *e.g.* 4 chemicals/year practically possible (laboratory issue)?

We need to evaluate the assay validity with both data from the on going validation studies

and the published studies (retrospective validation), *i.e.*, prospective and retrospective validation. We need to compile and review such published data. However, the current protocol is not fit to the old data, and we should determine acceptable criteria for the old data, *e.g.*, to check conformity to published recommendations (Tice, et al., 2000, Hartmann, et al., 2003, Burlinson, et al., 2007).

Regarding the main studies, there were opinions as follows: the current trial will be enough to show the reproducibility, and if the present validation study goes well, then one chemical per each laboratory may be sufficient to evaluate the assay sensitivity/specificity; we can test twice chemicals, which may be much more important.

Regarding acceptability of new laboratory (historical data + one chemicals testing), VMT members discuss in the luncheon VMT meeting.

Again, how many chemicals should we evaluate (VMT issue)? To examine the assay specificity is more important (use of in vitro +ve non-carcinogens), and not so many typical genotoxic agents will be included. Practical chemical selecting should be based on the modes of action. COLIPA has a chemical list for assay validation, and we may refer to it.

Regarding possibility of incorporation with general toxicity studies (considering ICH S2(R)), we need to think about it after the main validation studies. It may be enough to use very limited number of chemicals to be evaluated for the purpose.

4. Luncheon VMT meeting (member: Hayashi, Hartung, Schechtman, Tice, Uno)

VMT members discussed how to select laboratories for the main studies. For examination, 2 chemicals, i.e., +ve and –ve chemicals, should be tested in a candidate laboratory. For less experienced laboratories, dose-response tests on the +ve control chemical (EMS) should be tried twice to confirm the reproducibility, and then +ve and –ve chemicals should be tested as for experienced laboratories. Technical transfer is important and we need to seek the possibility to have it at three regions.

5. Protocol of *in vitro* pre-validation study

Dr. Uno reported the summary of in vitro comet assay meeting on August 24 (see the meeting minute). Assay sensitivity of *in vitro* Comet assay was discussed again, and we need to continue the discussion. Following are candidates of leading laboratories: Anpyo, BioReliance, FDSC.

At the luncheon VMT meeting, members recommended to use two kinds of cell lines for the in vitro comet assay validation study.

Draft Minutes
4th meeting of the International Validation study
On Comet Assay
Date: March12-14, 2008
(March 12, 9:00 to 17:00, March 13, 9:30 to 17:00, March 14, 8:30 to 15:00)
Venue: Atagawa hights,
Shizuoka, Japan

M. Hayashi (NIHS), Y. Uno (Mitsubishi Tanabe Pharma Co.), M. Honma (NIHS), H. Kojima (JaCVAM, NIHS), N. Asano (Nitto Denko Co. : MMS president), T. Morita (NIHS), M. Nakajima (Anpyo-Center), K. Yamakage (FDSC), Y. Nakagawa (FDSC), J. Tanaka (Anpyo-Ceneter), M. Ueda (Anpyo-Center), T. Omori (Kyoto Univ.), A. Kimura (NIHS), N. Tanaka (FDSC), A. Sakai (FDSC), B. Burinson (Huntingdon Life Science), P. Escober (Boeringer-ingelheim), D. Lovel (Univ. of Surrey), A. Kraynak (Merck), Young Na Yum (KFDA), R. Corvi (ECVAM), S. Hoffman (ECVAM), K. Pant (BioReliance)

1

March 12, 2008/Day 1/In vitro comet 1

- This section was leaded by Dr. M. Honma
- Dr. Hayashi addressed welcome remarks and Dr. Kojima gave hose keeping announcement.
- Dr. Honma leads today
- Dr. Honma showed his data (his laboratory is not a leading laboratory)
  - S9 problem
  - Treatment time was not critical robust for the treatment time
    - 4 h had no problem
  - Cytotoxicity
    - Trypan blue and RCG
  - Comet assay itself is as same as for in vivo assay
    - Duplicate culture to show the reproducibility

2

March 12, 2008/Day 1/In vitro comet 2

- Test chemicals used were same for the prevalidation
  - All chemicals were delivered by the VMT
  - Same lot of chemicals
- Preliminary experiment from Honma's laboratory (see 8.2)
- Honma's lab did not take duplicate culture (some simplified protocol)
  - TB method was useless direct after treatment
  - He explained the data (8.2) together with the MN data
  - MMC, no response at all, not reduced because the control value was too low to show the reduction

3

March 12, 2008/Day 1/In vitro comet 3

- S9 mix did not work properly in the comet assay even cylclophosphamide
  - Cytotoxicity was not enough strong
  - Neither MN induction
  - Kikkoman S9 worked/specification was almost equivalent between French (Biopredic) and Kikkoman
  - Merck worked
  - BR not worked in the first exptl and worked the following exptl
- TB cytotoxicity test was commonly used in the mammalian assay because everyone thought it was the easy way. Dr. Uno suggest that we need the selection of the cytotoxicity assay more carefully. We will discuss later after other laboratories presentation.

4

March 12, 2008/Day 1/In vitro comet 4

- BR presentation (8.4 short version)
  - Diffrences form the standard protocol
    - Normal agarose from bottom gel
    - Cell washed and re-suspended in chilled complete media electrophoresis at room temp (21 2C and 2-8C)
  - RCG (cell count and TB) and ATP for cytotoxicity: both showed similar and reliable response)
  - Neutral-diffusion assay was not successful: subjective and no information about cytotoxicity: no difference between patterns of B and C and suddenly NA (no cell) [triton X].
  - 23.67 vs 4.17 in ethanol between duplicate culture. Merck has also similar phenomenon. One explanation is scoring. Blinding, prefixed area to analyzed. Subjectivity. Density of cell in the agar might be important to get more consistent data. Sample preparation might be important: slide quality.
  - % tail showed big difference but tail migration???
  - Acceptance criteria is important and should be set.

5

March 12, 2008/Day 1/In vitro comet 5

- Merck presentation
  - No difference from the standard protocol
- Hedgehogs in control group showed 2-7%; ethanol 17, 17
- Some positive response were observed in MMC
- TB for cytotoxicity showed no sense response
- 2AA specific figure (hedgehog), core in the cloud
- Hedgehog can provide good information about cytotoxicity

6

March 12, 2008/Day 1/In vitro comet 6

- Huntingdon presentation
  - EMS showed good dose response positive but top two conc showed too many hedgehogs
  - Again, TB 0 day showed no sense for cytotoxicity. RCG was good indication.
  - MMC, at low conc increase comet but higher concs decrease comet dose-depending manner (control level was 14.22%): bell-shape curve
  - One example big difference between duplicate in % tail but not so big in tail length
  - 2-amino anthracene showed too cytotoxic in the presence of S9 mix (without S9 mix also showed less convincing outcomes; precipitation, hedgehogs). This chemical will be repeated.
  - Variation of control % tail (about 5 to 16%) cross studies

7

March 12, 2008/Day 1/In vitro comet 7

- Cell differences
  - TK6 cell has many advantages. E.g., human cell line, with normal P53 situation.
  - L5178Y cell has also advantage together with gene mutation assay
  - Human lymphocytes; variability among donors: low background
  - BR: although there are some differences we can use any cell types
- Why not include positive control in all experiments?
  - Because at the moment, we used clear positive and negative chemicals and no need positive control group.

8

March 12, 2008/Day 1/In vitro comet 8

- Hadano presentation
  - 2AA different conc was used. Other condition were followed the standard protocol
- Again TB immediately after treatment did not show any response
- S9, sometimes works but not always
- Cyclophosphamide needs higher conc to induce comet compare to the CA assay
- In vivo, comet and MN response are observed under the similar dose levels but not in vitro. In vitro, comet usually require higher dose compare to the MN assay.

9

March 12, 2008/Day 1/In vitro comet 9

- Anpyo presentation (only one chemical) EMS
- Again, TB 0 day did not work.

10

March 12, 2008/Day 1/In vitro comet 10

- Dr. Honma explain the standard data sheet to be used in the in vitro validation study
- 2 slide from 1 culture
- Dr. Suzuki is taking care of this.

11

March 12, 2008/Day 1/In vitro comet 11
To be discussed

- How to asses cytotoxicity
  - Endpoint/Treatment protocol
    - TB direct after treatment is no longer necessary.
    - TB 3-4 h after treatment can be used
    - RSG 24 h should be included
    - ATP assay has some limitations (optional use)
    - Neutral diffusion is not good indicator for cytotoxicity
- How to overcome inconsistency between duplicate cultures
  - Density of cells in agar
  - How to reduce subjectivity
    - Blinding
    - Automation without man decision to select cells to be analyzed
    - In vitro and in vivo issues
- Technical transfer, quality control of preparations
  - More detail protocol

12

March 12, 2008/Day 1/In vitro comet 12
To be discussed

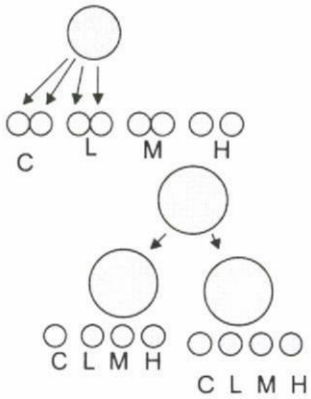
- To be discussed
  - Hedgehog
  - Special issue for in vitro assay. It is good indication of slide quality as well as cytotoxicity. Definition of hedgehog and comet: machine dependent. Another scan to count hedgehog among 100 cells.
- Cell type; TK6 is good enough or we have to think about the other cell types
  - Although TK6 is not perfect, we keep to use TK6 for the validation study
- Positive control
  - EMS as same as in vivo comet assay (250 μg/mL) in this validation study (expectation response: minimum 2 folds in % tail)
- S9
  - We have to try again using DMN and CYP (only with S9 mix).
    - 3-4 h treatment; MolTox S9 will be used
- Serum
  - FBS is recommended in the validation study.

13

March 12, 2008/Day 1/In vitro comet 13
To be discussed

- Positive definition; what we want to detect
  - Both in vitro and in vivo
- Duplicate culture
  - At least duplicate culture. To analyze the present control intra- and inter-laboratories.
- Electrophoresis; e.g., temperature
  - Both in vitro and in vivo
- How to score slides
  - Both in vitro and in vivo

14



15

March 13, 2008/Day 2/In vivo comet 1

- This section was leaded by Dr. Y. Uno
- Dr. Omori delivered graphs of all outcomes from the phase 2 validation. All participants, without statistician, marked + or – for each graphs. These intuitional judgments will be looked into by statistician group meeting after lunch.
- Dr. Omori asked participating laboratories to check again all data they provided.
- Dr. Omori showed the summarized data and explained the outcomes.
- Dr. Uno broke the code of chemicals, A, B, and C.
- Dr. Uno asked each laboratory to present data briefly.
- L-1 Merck, 2-BR, 3-HLS, 4-Hadano, 5-Anpyo

16

March 13, 2008/Day 2/In vivo comet 2

- Dr. Omori explained using figures. E.g., trend test, 5% both sides and pair-wise comparison (Dunnett test) 5% one side with (J_D) and without (J_L) consideration of multiplicity.
- We have to discuss whether one side or two sides test should be used for further evaluation. E.g., should we consider to detect the cross-linking agent.
- Fig. 7L
- Dr. Uno explained the terminology:
  - Endpoint: individual values of each parameter
  - Estimate: a mean calculated with values of endpoint
  - Effect: difference of an average of estimate between a control group and a treatment group.

17

March 13, 2008/Day 2/In vivo comet 3

- Fist, only EMS data were discussed in effect at each laboratory
- Lab 2 showed big variance comparing to other laboratories
- Average inter-laboratory was approximately 100
- Variability between laboratories was approx 300
- Intra-laboratory variation varied from 0 to 400

18

March 13, 2008/Day 2/In vivo comet 4

- Stomach showed much more variability compared to Liver
  - Average inter-laboratory was approximately 100
  - Variability between laboratories was approx 300
  - Intra-laboratory variation varied from 0 to 300
- Fig. 9 showed the differences between liver and stomach

19

March 13, 2008/Day 2/In vivo comet 5

- Merck presentation
  - #1 slight increase in liver and stomach at low dose
  - #2 slight increase in liver and negative in stomach
  - #3 negative in both liver and stomach
  - #3 showed slight change in liver at all dose but not in stomach in the high dose group (NE lower dose levels)
- The timing of treatment might be important especially for the stomach

20