

Scantox Test Report Lab#26832, Edwards CN: Haematococcus pluvialis mouse
micronucleus test. pp 1-14, April 1998.



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TEST REPORT

Lab No 26832
Issued: 15.04.1998
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SPONSOR:

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HAEMATOCOCCUS PLUVIALIS

MOUSE MICRONUCLEUS TEST

AUTHOR:

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GLP COMPLIANCE

The investigation described in this report "Haematococcus pluvialis - Mouse Micronucleus Test" was carried out under my supervision and responsibility and in accordance with the principles of Good Laboratory Practice (GLP) according to OECD codes of GLP, May 1981, Doc C(81)30 (Final) Annex 2, which are essentially in conformity with:

EEC Principles of Good Laboratory Practice, Directive 87/18/EEC,
United States Food and Drug Administration, Title 21, CFR, part 58, and
Japanese Ministry of Health and Welfare, PAB Notification No. 313.

The report is a complete and accurate account of the methods employed and the data obtained.

SCANTOX
15.04.1998

C N Edwards

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QUALITY ASSURANCE STATEMENT

The Quality system at Scantox complies with the OECD principles of Good Laboratory Practice and the European Standards EN45001.

Short term routine studies of the type described in this report "Haematococcus pluvialis - Mouse Micronucleus Test" are inspected by the Quality Assurance Unit in compliance with the principles of Good Laboratory Practice. Process-based inspections are carried out regularly. Documented inspection reports are communicated to the study director and to the management.

Date of most recent inspection: 14.01.1998

Date of report to study director and management 14.01.1998

This report has been audited by the Quality Assurance Unit and was found to be an accurate description of the methods and procedures used during the conduct of the study and an accurate reflection of the raw data.

Date of final audit: 15.04.1998

15.04.1998



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SUMMARY

Haematococcus pluvialis was tested in the Mouse Micronucleus Test performed in accordance with the guideline recommended by OECD "Micronucleus Test", No 474, 1983. The test also fulfils the corresponding EEC guideline B12 (1992) for a 'screening' study.

Groups of five male and five female mice were dosed once orally by gavage with Haematococcus pluvialis at 2000 mg/kg, the maximum dose required by the OECD and EEC guidelines for materials of low toxicity. Distilled water was used as the vehicle and negative control. The dose volume was 10 ml/kg body weight. Cyclophosphamide (20 mg/kg) served as the positive control.

Bone marrow samples were taken 24 and 48 hours after dosing.

No statistically significant reduction in the frequency of polychromatic erythrocytes among total erythrocytes was observed after treatment with Haematococcus pluvialis, indicating that there was no toxic effect on the bone marrow.

No biologically or statistically significant increases in the frequency of micronucleated polychromatic erythrocytes were seen in mice treated with Haematococcus pluvialis.

A large, statistically significant increase in the frequency of micronucleated polychromatic erythrocytes was observed in the positive control group, demonstrating the sensitivity of the test system.

It is concluded that Haematococcus pluvialis showed no evidence of mutagenic/clastogenic activity in this mouse micronucleus test after administration by oral gavage at 2000 mg/kg.

INTRODUCTION

The mouse micronucleus test is a short term *in vivo* mutagenicity test for the evaluation of possible mutagenic (chromosome damaging) effects of chemicals. The test was performed in accordance with the guideline recommended by OECD "Micronucleus Test", No 474, 1983. The test also fulfils the corresponding EEC guideline B12 (1992) for a 'screening' study.

General description of the test system

The micronucleus test, as described by Schmid (1), is based on the observation that erythroblasts in the bone marrow expel their nucleus in the last stage of erythropoiesis to become polychromatic (immature) erythrocytes (PCE). Acentric chromosome fragments arising from chromosome breakage (and also single chromosomes detached from the mitotic spindle) will remain in the cell, thereby giving rise to micronuclei which can be observed using a microscope after staining.

A measure of the chromosome damaging effect of the test chemical is obtained by comparing the frequency of micronucleated PCE in the bone marrow of treated versus control animals.

Reason for choice of animal species and route of administration

The mouse was used since it is the most widely used species for this test. The oral route of administration was chosen for evaluation of the risk of human exposure.

The dose level (2000 mg/kg) was selected on the basis of the results obtained in a preliminary toxicity test in which it was found that no adverse reactions to treatment were observed in mice treated at 2000 mg/kg and sacrificed 48 hours later. This is the highest dose required by the OECD and EEC guidelines for materials of low toxicity.

The mice for the preliminary toxicity test arrived at Scantox on 2 January 1998. They were dosed on 7 January 1998 and killed on 9 January 1998. The mice for the main micronucleus test arrived at Scantox on 07 January 1998. They were dosed on 12 January 1998 and the last mice were killed on 14 January 1998. The slide scoring was completed on 19 February 1998.

MATERIALS AND METHODS

Test article and vehicle

Haematococcus pluvialis was received from the Sponsor on 23 December 1997 and stored at approximately -20°C in the dark. Test article characterization (purity, stability etc) was the responsibility of the Sponsor. The test article, batch 971215:6, was properly labelled with the Lab No of the study (Lab No 26832).

Sterile distilled water, batch No 97B05S03, was used as the vehicle.

The test results relate to the above mentioned test article supplied by the Sponsor.

Animals

Twenty-five male and twenty-five female SPF mice of the stock Bom:NMRI from Bornholtgaard Breeding and Research Centre A/S, DK-8680 Ry were used for the main micronucleus test. A further two males and two females were used for the preliminary toxicity test. The mice were allowed to acclimatise for 5 days after receipt before use. By the time of dosing the animals were about 7 weeks old and weighed 25 to 29 g. Extra mice were available until the day of dosing so that animals with body weights outside this range might be replaced. The animals were randomly assigned to control and test groups using a randomization scheme.

Housing

The study took place in animal room No 4 provided with filtered air at a temperature of $21 \pm 3^\circ\text{C}$, relative humidity of $55\% \pm 15\%$ and with 10 air changes/hour. The room was illuminated to give a cycle of 12 hours light and 12 hours darkness. The light was on from 06 to 18 h.

The temperature and relative humidity in the animal room were recorded hourly and the records are retained. During the study, the actual temperature and relative humidity remained within the ranges given above.

The mice were kept in single sex groups of two or five in transparent polycarbonate (macrolone type III) cages (floor area: 810 cm²).

Bedding

The bedding was softwood sawdust "Hahnflock H ¼" from Hahn & Co., D-24796 Bredenbek-Kronsborg. Regular analyses for relevant possible contaminants are performed. Certificates of analysis are retained.

Diet

A complete pelleted rodent diet "Altromin 1314" (for growing animals) from Chr. Petersen, DK-4100 Ringsted, was available *ad libitum*. Analyses for major nutritive components and relevant possible contaminants are performed regularly. Certificates of analysis are retained.

Drinking water

The animals had free access to bottles with domestic quality drinking water acidified with hydrochloric acid to pH 2.5 in order to prevent microbial growth. Analyses for relevant possible contaminants are performed regularly. Certificates of analysis are retained.

Animal and cage identification

The animals were dosed orally by gavage according to the scheme shown below. All test and control formulations were given in a volume of 10 ml/kg body weight.

<u>Group</u>	<u>No of animals</u>	<u>Treatment</u>	<u>mg/kg</u>	<u>Bone marrow sampling</u>
Control	5 M + 5 F	Vehicle	-	24 h after dosing
Control	5 M + 5 F	Vehicle	-	48 h after dosing
Test Group	5 M + 5 F	Test article	2000	24 h after dosing
Test Group	5 M + 5 F	Test article	2000	48 h after dosing
Positive Control	5 M + 5 F	Cyclophosphamide*	20	24 h after dosing

*Sendoxan®, ASTA Medica AG, Frankfurt am Main, Germany.

The cages were identified by cage cards marked with the study number (Lab No 26832), group number, and sex of the animals. The animals were marked with unique numbers on the tail.

Bone marrow preparation

Immediately after killing by dislocation of the neck, the right femur from each mouse was dissected free. The bone marrow was flushed out into 2.5 ml of foetal calf serum using a 1 ml syringe and needle. After vortex mixing, the suspension was centrifuged for 10 minutes at 1000 rpm and most of the supernatant was removed. The cells were resuspended in the remainder and smeared on clean glass slides. The specimens were fixed in methanol and stained with May - Grünwald/Giemsa (Merck). The slides were air-dried and coverslips were applied using Dammarxylen® mountant.

Microscopic analysis

Prior to microscopic analysis, one slide from each animal was given a code number by a person who was not involved in the microscopic analysis. The code labels covered all unique identification marks on the slides.

For each animal the following counts were made:

Number of polychromatic erythrocytes (PCE) per 1000 erythrocytes.

Number of micronuclei (Mn) in 1000 polychromatic erythrocytes.

Number of micronuclei (Mn) in normochromatic erythrocytes (NCE) observed during scoring of the 1000 erythrocytes.

After analysis of all slides the code was broken, and the data presented in tables.

Criteria for identifying micronucleated erythrocytes

A micronucleus was defined in the following way:

- A bluish mauve strongly coloured uniform circular particle in the cell
- The particle should have a certain size (not being punctiform) and it should be located in the same plane as the cell (the cell and the micronucleus should be in focus at the same time)
- During focusing the particle should stay uniform in colour/light refraction and shape within a relatively large interval
- Cells with two or more micronuclei are counted as single micronucleated cells.

Evaluation of results

The frequency of micronucleated polychromatic erythrocytes in the test and positive control groups was compared to the frequency found in the vehicle control group. Statistical analysis was performed using a one way Analysis of Variance based on rank values (Blom's method (2)).

The statistical analyses were made with SAS® procedures (version 6.12) described in "SAS/STAT® User's Guide, Version 6, Fourth Edition, Vol. 1+2", 1989, SAS Institute Inc., Cary, North Carolina 27513, USA and StatXact® procedures described in StatXact® Turbo User Manual, 1992, Cytel Software Corporation, Cambridge, MA 0139, USA.

Archives

For a period of 10 years the following material relating to the study will be retained in the archives of Scantox:

- Protocol, protocol amendments and correspondence
- Test material receipts
- All original data
- Specimens and slides
- Final report

At the end of the storage period Scantox will contact the Sponsor for instructions whether the material should be transferred, retained or destroyed.

RESULTS

Clinical signs and mortality

No adverse clinical signs were recorded during the study period and all animals survived to scheduled sacrifice.

Frequency of PCE and micronucleated PCE

The frequencies of PCE among total erythrocytes and the frequencies of micronucleated PCE from individual animals at the 24 and 48 hour harvest times are shown in Tables 1 and 2. A summary of the results and statistical analysis is presented in Table 3.

The positive and negative control values were within acceptable ranges.

No statistically significant effect of *Haematococcus pluvialis* was seen on the frequency of PCE among total erythrocytes, indicating that there was no toxic effect on the bone marrow.

No statistically significant increases in the frequency of micronucleated PCE were seen at either harvest time in mice treated with *Haematococcus pluvialis*.

CONCLUSION

It is concluded that *Haematococcus pluvialis* showed no evidence of mutagenic/clastogenic activity in this mouse micronucleus test after administration by oral gavage at 2000 mg/kg.

REFERENCES

- (1) W. Schmid, The micronucleus test, *Mut. Res.* 31, pp. 9-15 (1975).
- (2) G. Blom, *Statistical Estimates and Transformed Beta Variables*, New York: John Wiley and Sons, Inc. (1958).

Mouse micronucleus test with *Haematococcus pluvialis*

Individual results, 24 hour sampling time

Treatment	Animal number	Sex	Mn/PCE	Mn/NCE	%PCE
Vehicle control	1	Male	0	0	51
	2	Male	2	1	43
	3	Male	1	0	43
	4	Male	0	0	44
	5	Male	0	0	47
	6	Female	4	0	49
	7	Female	0	0	48
	8	Female	1	0	44
	9	Female	1	0	44
	10	Female	3	1	43
<i>Haematococcus pluvialis</i> 2000 mg/kg	21	Male	1	0	49
	22	Male	1	1	46
	23	Male	1	0	49
	24	Male	0	0	53
	25	Male	1	1	40
	26	Female	1	0	45
	27	Female	0	0	41
	28	Female	0	0	48
	29	Female	2	2	42
	30	Female	0	2	46
Positive control Cyclophosphamide 20 mg/kg	41	Male	21	1	45
	42	Male	43	4	41
	43	Male	32	2	42
	44	Male	19	4	41
	45	Male	24	4	43
	46	Female	24	3	43
	47	Female	14	1	38
	48	Female	29	2	39
	49	Female	39	2	40
	50	Female	15	1	41

Mn/PCE Number of polychromatic erythrocytes (PCE) with micronuclei (1000 PCE scored)

Mn/NCE Number of normochromatic erythrocytes (NCE) with micronuclei (1000 erythrocytes scored)

%PCE Frequency of PCE among total erythrocytes (%) (1000 erythrocytes scored)

Mouse micronucleus test with *Haematococcus pluvialis*

Individual results, 48 hour sampling time

Treatment	Animal number	Sex	Mn/PCE	Mn/NCE	%PCE
Vehicle control	11	Male	1	0	49
	12	Male	0	1	51
	13	Male	1	2	45
	14	Male	1	2	46
	15	Male	0	1	47
	16	Female	1	0	51
	17	Female	2	2	52
	18	Female	0	0	40
	19	Female	1	0	47
	20	Female	0	0	45
<i>Haematococcus pluvialis</i> 2000 mg/kg	31	Male	1	0	48
	32	Male	0	1	47
	33	Male	0	0	48
	34	Male	0	0	50
	35	Male	2	0	48
	36	Female	2	0	49
	37	Female	1	0	46
	38	Female	2	0	46
	39	Female	0	0	46
	40	Female	0	1	55

Mn/PCE Number of polychromatic erythrocytes (PCE) with micronuclei (1000 PCE scored)

Mn/NCE Number of normochromatic erythrocytes (NCE) with micronuclei (1000 erythrocytes scored)

%PCE Frequency of PCE among total erythrocytes (%) (1000 erythrocytes scored)