

## **Annex I to the CLH report**

### **Proposal for Harmonised Classification and Labelling**

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),  
Annex VI, Part 2**

### **International Chemical Identification: Hexyl salicylate**

**EC Number:** 228-408-6

**CAS Number:** 6259-76-3

**Index Number:** -

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## **1 PHYSICAL HAZARDS**

### **1.1 Explosives**

#### **1.1.1 Explosive properties**

*Test type*

Statement

*Detailed study summary and results:*

Hexyl Salicylate does not contain any groups associated with explosivity. Explosive properties are associated with the presence of certain chemical groups in a molecule which can react to produce very rapid increases in temperature or pressure. When there are no chemical groups associated with explosive properties present in the molecule then a substance or mixture shall not be classified as explosive.

*Material and methods*

NA

*Results*

No explosive properties

### **1.2 Flammable gases (including chemically unstable gases)**

Non applicable as the substance is a liquid.

### **1.3 Oxidising gases**

Non applicable as the substance is a liquid.

### **1.4 Gases under pressure**

Non applicable as the substance is a liquid.

### **1.5 Flammable liquid**

#### **1.5.1 Flammability**

*Study 1 reference:*

Rogers, G 2010 : Auto-Ignition and Flash Point Testing on a Sample of Hexyl Salicylate (study report), Testing laboratory: Chilworth Technology Limited Process Safety Laboratories Beta House Southampton Science Park Southampton SO16 7NS United Kingdom, Report no: GLP103396AR1V1/09. Study number: GLP/103396/A, Report date: Jan 13, 2010.

***Test type***

Flash point

***Detailed study summary and results:***

A flash point of 151 °C was recorded for Hexyl Salicylate. As Hexyl Salicylate is not a gas oil, diesel, light heating oil with flash point up to 75°C or a halogenated substance, mixture containing halogenated, volatile or non volatile flammable substance, it should not be subject to hazard class 'flammable liquid'.

***Results***

The flash point of hexyl salicylate was determined as 151 °C.

Hexyl salicylate is therefore not classified as flammable liquid.

## **1.6 Flammable solids**

Non applicable as the substance is a liquid.

## **1.7 Self-reactive substances**

### **1.7.1 Self-reactivity**

***Test type***

Statement

***Detailed study summary and results:***

Hexyl Salicylate does not contain any groups associated with self-reactivity. Self-reactive properties are associated with the presence of certain chemical groups in a molecule which can react to produce very rapid increases in temperature or pressure. When there are no chemical groups associated with self-reactive properties present in the molecule then a substance or mixture shall not be classified as self-reactive.

***Material and methods***

NA

***Results***

Not classified as self-reactive

## **1.8 Pyrophoric liquids**

### **1.8.1 Pyrophoricity**

***Test type***

Statement

***Detailed study summary and results:***

Hexyl Salicylate does not contain any groups associated with pyrophoricity, in particular it does not contain any metal or metalloid.

### ***Material and methods***

NA

### ***Results***

Not classified as pyrophoric.

## **1.9 Pyrophoric solid**

Non applicable as the substance is a liquid.

## **1.10 Self-heating substances**

Non applicable as the substance is a liquid.

## **1.11 Substances which in contact with water emit flammable gases**

### **1.11.1 Solubility in water**

#### ***Test type***

Solubility in water

#### ***Detailed study summary and results:***

The solubility of hexyl salicylate was determined to be 2 mg/L at 23 °C.

#### ***Results***

The solubility of hexyl salicylate was determined to be 2 mg/L at 23 °C. No reaction occurred, and in particular no gas is emitted in contact with water.

## **1.12 Oxidising liquids**

### **1.12.1 Oxidising properties**

#### ***Study 1 reference:***

Brady, D. 2010: Expert statement on the oxidizing properties of Hexyl Salicylate (CAS 6259-76-3) (study report), Testing laboratory: TSGE Concordia House St James Business Park Knaresborough North Yorkshire HG5 8QB UK, Report no: TSGE PLE.001 001 Oxidising. Report date: Jun 18, 2010.

#### ***Test type***

Statement

#### ***Detailed study summary and results:***

Considering the structural environment of oxygen in the molecule and the oxygen balance of Hexyl Salicylate (CAS: 6259-76-3), it can be concluded, beyond reasonable doubt, that Hexyl Salicylate (CAS: 6259-76-3) is unlikely to be an oxidizer and will be incapable of reacting exothermically with combustible materials. It need not be tested experimentally for oxidizing properties.

***Material and methods***

NA

***Results***

Not classified for oxidising properties.

**1.13 Oxidising solids**

Non applicable as the substance is a liquid.

**1.14 Organic peroxides**

**1.14.1 Organic peroxides**

***Test type***

Statement

***Detailed study summary and results:***

Hexyl salicylate is not classified as an organic peroxide as defined by its molecular structure.

***Material and methods***

NA

***Results***

Not classified as organic peroxide.

**1.15 Corrosive to metals**

**1.15.1 Corrosive to metals**

***Test type***

Statement

***Detailed study summary and results:***

The substance does not contain any halogen atom, has neither acidic nor alkaline functional groups, and is not known to form complexes with metals.

***Material and methods***

NA

***Results***

Not corrosive to metals

## 2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

### 2.1.1 Study report (2016)

#### *Study reference*

Study report. 2016. Untitled.

#### *Test type*

This study was an *in vitro* dermal absorption test performed according to OECD Guideline 428. This test was GLP compliant.

#### *Material and methods*

Human abdominal or breast skin membranes (thickness: 0.2-0.4 mm) (n=8) obtained from 4 female donors.

Exposure to 0.1, 20 and 100% radiolabelled hexyl salicylate (99.8% radiochemical purity, specific activity 61.3 mCi/mmol). Vehicle used : dipropylene glycol.

Principles of the assay: the test substance was applied to the surface of the skin sample separating two chambers (a donor chamber and a receptor chamber - physiological saline with 6% PEG 20 -) of a diffusion cell (9 mm automated flow-through cells) for 24h. The test temperature was 32 +/- 1°C. Exposure was terminated by washing at 8 hours with a 3% soap solution and the skin membranes were tape-stripped at termination of the study 24 hours after exposure. At each concentration of hexyl salicylate, the majority of the applied radioactivity was removed by washing at 8 hours (97.6%, 87.9% and 93.5% at concentrations of 100%, 20% and 0.1%, respectively). Only relatively small amounts of radioactivity (0.15%, 0.64% and 1.00%, respectively) were detected in the receptor fluid.

In a separate metabolism phase, <sup>14</sup>C-radiolabelled hexyl salicylate (0.1% in dipropylene glycol) was applied to breast or abdomen skin membranes (n=3) from two female donors using static diffusion cells and tissue culture medium as receptor fluid. The skin membranes were used shortly following receipt and without freezing, in order to maintain metabolic capacity.

#### *Results*

Dermal absorption values of 0.8%, 7.8% and 2.7% are calculated for hexyl salicylate concentrations of 100%, 20% and 0.1% respectively, taking into account the potential for metabolism to salicylic acid in viable skin. Detailed results are reported on the table below.

Summary of the dermal absorption phase

Group	A	B	C
Concentration of hexyl salicylate	100%	20%	0.1%
Number of replicates	8	8	8
Maximal flux (µg.cm <sup>-2</sup> .h <sup>-1</sup> )	0.84 ± 0.12	0.83 ± 0.21	0.007 ± 0.001
	Recovery (% of dose, mean±SD)		
Amount in receptor fluid	0.15 ± 0.02	0.64 ± 0.15	1.00 ± 0.16
Amount in receptor compartment wash	0.009 ± 0.001	0.072 ± 0.017	0.037 ± 0.018
Amount in (stripped) skin	0.38 ± 0.14	2.33 ± 1.32	1.30 ± 0.62
Amount in tape strips 1+2	0.12 ± 0.09	2.62 ± 1.76	0.12 ± 0.08
Amount in tape strips 3-last	0.12 ± 0.08	2.16 ± 1.11	0.24 ± 0.15
Amount in skin wash	97.6 ± 1.8	87.9 ± 4.3	90.0 ± 3.1
Total recovery	98.5 ± 1.9	97.6 ± 0.9	93.5 ± 2.0
Absorbed dose 1	0.53 ± 0.14	3.04 ± 1.43	2.34 ± 0.69
Potentially absorbed dose 2	0.65 ± 0.19	5.20 ± 2.41	2.58 ± 0.77

1: the absorbed dose is defined as the amount in the receptor fluid, the receptor compartment wash and skin membrane, excluding tape strips

2: the potentially absorbed dose is defined as the amount in the receptor fluid, the receptor compartment wash, the skin and *stratum corneum* (except for the first two tape strips)

### 3 HEALTH HAZARDS

#### 3.1 Skin sensitisation

##### 3.1.1 Animal data

###### 3.1.1.1 Unnamed (2006)

###### *Study reference*

Unnamed. (2006). Hexyl salicylate: Local Lymph Node Assay.

###### *Test type*

LLNA assay. Equivalent or similar to OECD Guideline 429. GLP compliant.

###### *Test substance*

- Hexyl salicylate
- Analytical purity: 98.5%
- Batch number: No. 2005146-0012

###### *Test animals*

- Groups of four CBA female mice
- Age and weight at the study initiation: 8-12 weeks

###### *Administration/exposure*

- Concentrations tested: 1, 2.5, 5, 10, 25% (experiment 1) and 0.05, 0.25, 0.5, 1, 2.5% (experiment 2)
- Vehicle: 1:3 ethanol:diethylphtalate
- Positive control substance: hexyl cinnamic aldehyde (CAS No 101-86-0)
- Groups of 4 CBA mice were treated with the test material or vehicle alone on the dorsum of both ears. Treatment was performed once daily for three consecutive days. Three days after the third application, all mice were injected via the tail vein with approximately 250 µl phosphate buffered saline (PBS) containing 20 µCi of a 2.0 Ci/mmol specific gravity 3H-methyl thymidine. Mice were killed 5 hr later and the draining auricular lymph nodes excised and pooled for each experimental group. A single cell suspension of lymph node cells was prepared by mechanical disaggregation of lymph nodes through a 200-mesh stainless steel gauze. The cell suspensions were then washed three times by centrifugation with approximately 10 mL of PBS. Approximately 3 ml of 5% w/v trichloroacetic acid was then added and after overnight precipitation at 4°C, the samples were pelleted by centrifugation and the supernatant was discarded. The cells were then resuspended in approximately 1 ml of TCA. The lymph node suspensions were transferred to scintillation vials and 100 ml of scintillant optiphase was added prior to beta-scintillation counting.

**Results and discussion**

Hexyl Salicylate in 1:3 Ethanol:DEP was a skin sensitiser under the conditions of the test with an EC3 value of 0.18% (45 ug/cm<sup>2</sup>).

The detailed results are expressed as DPM per lymph node for each group in the table below.

Concentrations	DPM	Stimulation index
0 (vehicle only)	4737	1
1% w/v	47194	9.96
2.5% w/v	36531	7.71
5% w/v	69591	14.69
10% w/v	65268	13.78
25% w/v	109201	23.05
Repeat Test		
0 (vehicle only)	5464	1
0.05% w/v	10227	1.87
0.25% w/v	19466	3.56
0.5% w/v	30613	5.60
1% w/v	59186	10.83
2.5% w/v	59015	10.80

**3.1.1.2 Sharp (1978), cited in Lapczynski *et al.* (2007)****Study reference**

Lapczynski A, Jones L, McGinty D, Bhatia S, Letizia CS, Api AM. 2007. Fragrance material review on hexyl salicylate. Food Chem Toxicol.;45 (2007):S410-417. Epub 2007.

Review report.

Original study: Sharp, D.W., 1978. The sensitization potential of some perfume ingredients tested using a modified Draize procedure. Toxicology 9, 261–271.

**Test type**

Hexyl salicylate was tested in a guinea pig sensitization study using a modified Draize procedure in 10 inbred Hartley albino guinea pigs with initial weights of approximately 350 g each. Induction consisted of four intradermal injections with 0.1 ml of hexyl salicylate at 2.5 times the ICC (Injection Challenge Concentration = 0.1%) at four sites overlying the two axillary and the two inguinal lymph nodes. The animals were challenged 14 days later with an intradermal injection in one flank and a topical application in the other flank using 0.1 ml hexyl salicylate at 0.1% (ICC) and 5% (ACC), respectively. A second challenge was conducted 7 days later.

**Results**

Sensitization reactions were observed after the second challenge at 5%.

**3.1.1.3 RIFM (1981) cited in Lapczynski *et al.* (2007)****Study reference**

Lapczynski A, Jones L, McGinty D, Bhatia S, Letizia CS, Api AM. 2007. Fragrance material review on hexyl salicylate. Food Chem Toxicol.;45 (2007):S410-417. Epub 2007.

Review report.

Original study: RIFM (Research Institute for Fragrance Materials, Inc., 1981. Guinea Pig Skin Sensitization Test with Hexyl Salicylate. Report Number 46933, December 11 (RIFM, Woodcliff Lake, NJ, USA). Unpublished data.

### ***Test type***

A Magnusson–Kligman guinea pig maximization test was conducted on ten albino Dunkin/Hartley strain guinea pigs weighing 440–554 g. Induction consisted of intradermal injection followed one week later by a 48 h occluded patch. The six intradermal injections were made to a 2 x 4 cm clipped, shaved area in the dorsal shoulder region. There were two 0.1 ml injections of 1% hexyl salicylate in 0.01% DOBS/saline, two 0.1 ml injections of 1% hexyl salicylate in 50% Complete Freund's Adjuvant, and two 0.1 ml injections of 50% Complete Freund's Adjuvant. Seven days later, the site was clipped and shaved, and induction was supplemented topically with a 48 h occluded patch with 40% hexyl salicylate in acetone over the shoulder injection sites. Thirteen to 14 days after application of the shoulder patch, the guinea pigs were challenged on the clipped and shaved flank using an 8 mm diameter filter paper patch saturated with 10% hexyl salicylate in acetone which was applied for 24 h under occlusion. Reactions were assessed at 24 and 48 h after patch removal. Three additional challenge applications with 10% hexyl salicylate in acetone were made at weekly intervals on the contralateral flanks.

### ***Results***

No sensitization reactions were observed.

#### **3.1.1.4 RIFM (2003) cited in Lapczynski et al. (2007)**

##### ***Study reference***

Lapczynski A, Jones L, McGinty D, Bhatia S, Letizia CS, Api AM. 2007. Fragrance material review on hexyl salicylate. *Food Chem Toxicol.*;45 (2007):S410-417. Epub 2007.

Review report.

Original study: RIFM (Research Institute for Fragrance Materials, Inc., 2003. Topical Photoallergy Screening Test of Hexyl Salicylate in Male Albino Hairless Guinea. Report Number 44882, June 9 (RIFM, Woodcliff Lake, NJ, USA).

##### ***Test type***

The sensitization potential of hexyl salicylate was evaluated during a photoallergy test using Crl:IAF(HA)-hrBR outbred albino hairless guinea pigs (5/group). During the induction phase, an intradermal injection with a 0.1 ml aliquot of a formulation of sterile water and Freund's complete adjuvant (FCA) (1:1 v/v) was made to a 2.5 cm<sup>2</sup> nuchal area of skin. The skin area was then tape-stripped five times. For the topical induction, a 0.3 ml aliquot of 100% hexyl salicylate in 3:1 DEP:EtOH was applied using 25 mm Hilltop® chamber patches for 2 h. After patch removal, the application sites were gently wiped with disposable paper towels moistened with osmosis membrane-processed deionized water. This procedure was repeated on days 3, 5, 8, 10 and 12 of the induction phase. On day 22, the animals were topically challenged with 50% in 3:1 DEP:EtOH and 100% using the same procedure. The test sites were observed at 1 and 4 h, and at 1, 2 and 3 days after hexyl salicylate application.

##### ***Results***

No sensitization reactions were observed.

#### **3.1.2 Human data**

##### **3.1.2.1 RIFM (2004a), cited in Lapczynski et al. (2007)**

Lapczynski A, Jones L, McGinty D, Bhatia S, Letizia CS, Api AM. 2007. Fragrance material review on hexyl salicylate. *Food Chem Toxicol.*;45 (2007):S410-417. Epub 2007.

Review report.

Original study: RIFM (Research Institute for Fragrance Materials Inc.), 2004a. Repeated Insult Patch Test with Fragrance Materials. RIFM Report Number 45130, May, 3 (RIFM, Woodcliff Lake, NJ, USA).

### ***Test type***

A human repeated insult patch test (HRIPT) was conducted in 103 male and female volunteers (29 males/74 females) with 30% hexyl salicylate in 3:1 DEP:EtOH. Nine induction patch applications were made, three per week over a three week period. Each patch remained in place for 24 hours and reactions were assessed on removal. The patch consisted of a webril/adhesive patch (25 mm Hill Top Chamber), providing occluded covering. 0.3 mL of the test material was applied to the Hill Top Chamber. Each site was wiped with tissue to remove residual material after removal of the patch. The left side of the dorsum was used as the induction application site - each patch was placed on the same location for the nine induction applications. After a rest period of approximately 2 weeks, the right side of the dorsum, naive sites, was used for challenge. As in the induction phase, patches were applied for 24h. The test sites were also scored at 48, 72, and 96 h post-patching.

### ***Results***

No sensitization reactions were observed.

### **3.1.2.2 RIFM (1975b), cited in Lapczynski *et al.* (2007)**

Lapczynski A, Jones L, McGinty D, Bhatia S, Letizia CS, Api AM. 2007. Fragrance material review on hexyl salicylate. Food Chem Toxicol.;45 (2007):S410-417. Epub 2007.

Review report.

Original study: RIFM (Research Institute for Fragrance Materials Inc.), 1975b. Report on human maximization studies. RIFM report number 1798, January 31 (RIFM, Woodcliff Lake, NJ, USA). ***Test type***

A maximization test was carried out on 22 selected healthy volunteers from a group of 30 subjects using 3% hexyl salicylate in petrolatum. An occluded patch with hexyl salicylate was applied to the same site on the volar forearms or backs of each subject for five alternate 48-h periods. Patch sites were pre-treated for 24h with 5% aqueous SLS under occlusion. Following a 10–14-day rest period, a challenge patch was applied to a fresh site for 48h under occlusion. An application of 2% aqueous solution of SLS under occlusion was applied on the left side of the back for 30 min prior to challenge. Hexyl salicylate was applied without SLS on the right side. Reactions were read at patch removal and 24 h later.

### ***Results***

No positive reaction was produced. Biopsies of equivocal initial responses, followed by re-challenge, also produced no positive evidence of sensitisation.

### **3.1.2.3 Larsen *et al.* (2002), cited in Lapczynski *et al.* (2007)**

Lapczynski A, Jones L, McGinty D, Bhatia S, Letizia CS, Api AM. 2007. Fragrance material review on hexyl salicylate. Food Chem Toxicol.;45 (2007):S410-417. Epub 2007.

Review report.

Original study: Larsen W, Nakayama H, Fischer T, Elsner P, Frosch P, Burrows D, Jordan W, Saw S, Wilkinson J, Marks J, Sugawara M, Nethercott M, Nethercott J. 2002. Fragrance contact dermatitis – a worldwide multicentre investigation (Part III). Contact Dermatitis 46, 141-144.

### ***Test type***

In a multicenter study, 218 fragrance sensitive patients with proven contact dermatitis were patch tested with various fragrance materials according to internationally accepted criteria.

### **Results**

No reactions were observed with 5% hexyl salicylate in petrolatum.

#### **3.1.2.4 Bennike et al. (2019)**

##### **Study reference**

Bennike NH, Zachariae C, Johansenn JD. 2019. Optiman patch test concentration for three widely used sensitizing fragrance substances without mandatory labelling in cosmetics. *Contact Dermatitis*. 2019;80:325-327.

##### **Test type**

The study aimed at determining the optimal patch test concentration for 3 fragrance substances, including hexyl salicylate, by using a protocol published by the European Society of Contact Dermatitis. Approximately 100 consecutive dermatitis patients were patch tested with a starting concentration of 5% hexyl salicylate. This concentration was based on a previous clinical report on 218 fragrance-sensitized volunteers patch tested at a 5% concentration without any positive reactions (Larsen et al. 2002). Interim evaluations of the patch test results for these determined whether the individual concentrations were increased (by 50%) or decreased (by 33%) in the next series of ~100 patients. The concentration was decreased if active sensitization was expected or more than a few irritant reactions were registered. This procedure was repeated up to a maximum of four times. For the patch test procedure, 20 mg of test material suspended in pet. was applied to the upper back in Finn Chambers (8 mm; SmartPractice, Phoenix, Arizona), with an occlusion time of 2 days. Patch test readings were performed on day (D) 2, D3 and D4, and D5 and D7, and scored according to guidelines.

##### **Results**

No positive reactions to hexyl salicylate were seen.

## **3.2 Reproductive toxicity**

### **3.2.1 Adverse effects on sexual function and fertility**

#### **3.2.1.1 FDA (2006a)**

##### **Study reference:**

FDA. 2006. Center for Drug Evaluation and Research. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% 1-menthol Topical patch). NDA number 22-029. December 13, 2006

The description below is taken from Annex I to the CLH Report of methyl salicylate.

##### **Detailed study summary and results:**

##### **Test type**

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

##### **Test substance**

- Methyl salicylate

- Lot Y096
- 100.1%

### ***Test animals***

- Rats/Crj:CD(SD)IGS
- 20/sex/group for main study + 3/sex/group for toxicokinetics (satellite groups)

### ***Administration/exposure***

- 30, 100, 300 mg/kg/day. The high dose was based on the 2-week repeated dose preliminary study showing a depressive trend in body weight gain in males receiving 300 mg/kg and a decrease in food consumption in males and females receiving 300 mg/kg. The middle and low doses were set in a common ratio of about 3.
- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepared for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within  $100 \pm 5\%$ )
- The test article mixture was administered to the dorsal subcutis using a needle (26G) and syringe once daily from 2 weeks prior to mating through the mating period and up to day 6 of gestation for the females beginning at 10 weeks of age. The administration period before copulation in the males was determined as 2 weeks, since methyl salicylate did not show any testicular toxicity in the 2-week repeated dose preliminary study (dosage: 30, 100, 300 mg/kg).

### ***Description of test design:***

- Females aged 12 weeks were housed overnight with males aged 12 weeks in a 1:1 ratio. Copulation was confirmed by the presence of a vaginal plug or sperm in the vaginal smear on the following morning and the day of confirmed copulation was designed as day 0 of gestation. Mating was conducted within the same group for a maximum of 2 weeks. As for pairs for which copulation was unsuccessful, the males were mated with non-treated females and the females were mated with successfully copulating males in the same group during a maximum of 1 week.
- Parameters: clinical observation (clinical signs and mortality twice daily during administration period and once daily during other periods), body weight and food consumption (twice weekly and daily for females during gestation), estrous cycle (every morning), reproductive ability test (days required for successful copulation, copulation index, male and female fertility indices), necropsy (testes, left epididymis, ovaries, uterus and skin of the treated site) and organ weight (testes and epididymides), sperm (at necropsy, sperm count, sperm motility, sperm form anomalies index), numbers of corpora lutea implants, dead and live embryos, pre-implant loss index, dead embryo index.
- Determination of plasma salicylic acid concentration: blood was collected 1 time at 4 hours after administration on day 0 (first day) and 13 (final day) of administration. The concentration of salicylic acid was measured by HPLC.

### ***Results and discussion***

- Statistical analysis: mean and standard deviations with regard to body weight, body weight gain, food consumption, organ weight, estrous cycle, count of estrus, number of days required for successful copulation, sperm count and numbers of corpora lutea, implants and live embryos were calculated for every group and the homogeneity of variance was tested by Bartlett's method.

Comparison of the treated groups with the control group was made by Dunnett's method when the variance was found to be homogeneous or by Steel's method when the variance was not homogeneous. The copulation index and male and female fertility indices were analysed by  $\chi^2$  test. The sperm motility, sperm form anomalies index, pre-implant loss index and dead embryo index by Wilcoxon's rank sum test. Levels of  $P < 0.01$  and  $P < 0.05$  were considered to be significant in all cases.

*Effects on parent animals*

- One male in the 300 mg/kg group showed hypoactivity, bradypnea, hypothermia and blanching on day 3 of administration and died on day 4 of administration. In addition, crust on the treated site and/or loss of hair were observed in 2 females in the 300 mg/kg group from day 9 of administration to day 13 of gestation. There were no mortality or abnormal signs in the males and females in the control, 30 and 100 mg/kg groups.
- A significant lower body weight as compared with the control group was observed throughout the administration period in the males in the 300 mg/kg group and on days 1-3 and 10-14 of administration and throughout the gestation period in the females in the 300 mg/kg group.
- A significant depression of body weight gain as compared with the control group was observed on day 1-49 of administration in the males in the 300 mg/kg group ( $\geq -20\%$ ) and on days 10-14 of administration in the females in the 300 mg/kg group. During the gestation period, a significant depression of body weight gain as compared with the control group was observed on days 5 and 9-13 of gestation in the females in the 300 mg/kg group.

Day of Treatment	Vehicle Control	Mean Body Weight Gain (g) $\pm$ SD in Males in the High Dose Group	
		300 mg/kg/day	% Change of Control
1	5.3 $\pm$ 3.7	-6.3 $\pm$ 7.7**	$\downarrow$ 219%
3	17.4 $\pm$ 4.7	-3.1 $\pm$ 19.8**	$\downarrow$ 117.8.0%
7	40.0 $\pm$ 7.9	16.1 $\pm$ 13.4**	59.8%
10	55.4 $\pm$ 11.0	29.2 $\pm$ 16.0**	47.3%
14	74.7 $\pm$ 13.1	47.8 $\pm$ 16.5**	36.0%
17	83.4 $\pm$ 14.0	52.9 $\pm$ 18.7**	36.6%
21	97.7 $\pm$ 16.7	68.6 $\pm$ 19.9**	29.8%
24	114.5 $\pm$ 17.3	83.4 $\pm$ 21.9**	27.2%
28	126.3 $\pm$ 95.3	95.3 $\pm$ 24.9**	24.5%
31	140.7 $\pm$ 20.6	107.8 $\pm$ 26.1**	23.4%
35	153.4 $\pm$ 21.7	120.5 $\pm$ 30.8**	21.0%
38	163.3 $\pm$ 24.0	126.6 $\pm$ 31.9**	22.5%
42	174.9 $\pm$ 24.0	137.4 $\pm$ 32.1**	21.4%
45	182.3 $\pm$ 24.1	145.5 $\pm$ 34.9**	20.2%
49	194.1 $\pm$ 24.2	154.9 $\pm$ 37.0*	20.2%

\*\* Significantly different from vehicle control ( $p < 0.01$ )

Table 8 Body weight gains in female rats

Group and dose	Vehicle control		30 mg/kg		100 mg/kg		300 mg/kg	
Days of treatment	Body weight gain (g)							
1	0.3 $\pm$	6.3 (20)	-2.8 $\pm$	5.6 (20)	-5.2 $\pm$	6.1* (20)	-7.8 $\pm$	7.1** (20)
3	4.5 $\pm$	8.3 (20)	4.4 $\pm$	5.5 (20)	3.0 $\pm$	5.2 (20)	-4.9 $\pm$	12.8* (20)
7	13.8 $\pm$	8.0 (20)	14.3 $\pm$	6.4 (20)	10.8 $\pm$	6.6 (20)	11.2 $\pm$	7.1 (20)
10	22.7 $\pm$	9.8 (20)	20.8 $\pm$	8.9 (20)	16.9 $\pm$	8.2 (20)	13.8 $\pm$	11.6* (20)
14	28.8 $\pm$	10.9 (20)	28.9 $\pm$	10.3 (20)	23.8 $\pm$	9.2 (20)	19.0 $\pm$	12.2* (20)

\*:  $P < 0.05$ , \*\*:  $P < 0.01$  (significantly different from vehicle control). Values are mean  $\pm$  S.D. and the values in parentheses represent the number of animals.

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Table 9 Body weight gains in Fo dams

Group and dose		Vehicle control		30 mg/kg		100 mg/kg		300 mg/kg					
		Body weight gain (g)											
Days of gestation	1	6.7±	4.9	( 20)	8.9±	2.5	( 18)	6.7±	4.1	( 19)	7.9±	3.5	( 19)
	2	12.8±	4.0	( 20)	15.1±	4.2	( 18)	13.7±	4.0	( 19)	13.3±	4.8	( 19)
	3	19.1±	4.7	( 20)	19.9±	3.9	( 18)	19.6±	5.1	( 19)	19.3±	6.3	( 19)
	4	24.2±	4.6	( 20)	26.5±	4.8	( 18)	24.1±	6.2	( 19)	22.9±	6.7	( 19)
	5	29.9±	6.4	( 20)	31.2±	4.8	( 18)	27.9±	6.7	( 19)	24.8±	6.3*	( 19)
	6	34.9±	6.7	( 20)	34.5±	6.3	( 18)	32.4±	6.3	( 19)	31.5±	5.6	( 19)
	7	40.2±	6.6	( 20)	42.3±	6.8	( 18)	37.4±	6.9	( 19)	35.9±	7.6	( 19)
	8	45.5±	6.6	( 20)	47.0±	6.4	( 18)	43.5±	8.1	( 19)	41.7±	7.2	( 19)
	9	51.7±	8.3	( 20)	51.8±	7.0	( 18)	46.9±	8.3	( 19)	44.4±	7.3*	( 19)
	10	56.9±	8.5	( 20)	56.9±	8.5	( 18)	51.2±	9.2	( 19)	50.1±	8.3*	( 19)
	11	63.4±	9.5	( 20)	64.1±	7.6	( 18)	58.3±	8.7	( 19)	54.5±	8.7**	( 19)
	12	68.1±	10.0	( 20)	69.3±	8.2	( 18)	62.0±	9.9	( 19)	59.7±	8.9*	( 19)
	13	74.3±	9.4	( 20)	74.3±	9.0	( 18)	68.3±	10.2	( 19)	66.2±	8.2*	( 19)

#: P<0.05, \*\*: P<0.01 (significantly different from vehicle control).  
Values are mean±S.D. and the values in parentheses represent the number of dams.  
Four animals (30 mg/kg:2, 100 mg/kg:1 and 300 mg/kg:1) were non-pregnant.

- A significant decrease in food consumption as compared with the control group was observed in the 300 mg/kg group: in males on day 1 of administration (-27%) and in females on days 1 (-28.7%) and 3 (-17.1%) of administration and days 4 and 5 of gestation (-9 and -11%). Significant increase of food consumption as compared with the control group was observed on day 7 of administration in the 300 mg/kg group but this increase was considered to be incidental change not related to methyl salicylate administration since the change was transient.
- In necropsy of the males, retention of an oily fluid in the subcutis of the treated site was observed in all the males in the control and methyl salicylate groups. Dark red spot in the thymus and dark red macule in the subcutis of the treated site was observed in the dead male in the 300 mg/kg group. In necropsy of the dams on day 13 of gestation, retention of an oily fluid in the subcutis of the treated site was observed in all the dams in the control and methyl salicylate groups and in the non-pregnant females in the 30, 100 and 300 mg/kg groups. Crust of the treated site or loss of hair was observed in 2 dams each in the 300 mg/kg group.
- No significant differences in the weights of the testes or epididymides were observed in the methyl salicylate groups as compared with the control group.
- There were no significant differences between the control and methyl salicylate groups in the count of estrus or estrous cycle.

<b>Mean Count of Estrous ± S.D.</b>	3.70 ± 0.47	3.70 ± 0.73	3.90 ± 0.31	3.70 ± 0.80
<b>Mean Estrus Cycle Length ± S.D.</b>	4.10 ± 0.24	4.0 ± 0.0	4.13 ± 0.56	4.46 ± 1.83

- At the first mating, copulation was not confirmed in 1 pair each in the 100 and 300 mg/kg groups and 2, 1, 1 animals in the 30, 100 and 300 mg/kg groups, respectively, were sterile after copulation. Accordingly, the copulation indices were 100, 100, 95.00 and 94.74% for control, 30, 100, 300 mg/kg groups, respectively, and the male and female fertility indices were 100, 90.00, 94.74 and 94.44% for the control, 30, 100 and 300 mg/kg groups, respectively, results showing no significant difference between the control and methyl salicylate groups. No significant differences were observed between the numbers of days required for copulation by the control and methyl salicylate groups. No significant differences were observed between the numbers of days required for copulation by the control and methyl salicylate groups. When one male each in the 100 and 300 mg/kg groups in which copulation was not observed were mated with non-treated females, fertility was confirmed in both cases. When one female each in the 100 and 300 mg/kg groups in which copulation was not observed and in 1 female in the 300 mg/kg group whose paired male had died were mated with males in the same group that were confirmed to have copulated, fertility was confirmed in all cases.
- There were no significant differences between the control and methyl salicylate groups in the sperm form anomalies index, sperm count or sperm motility.

Dose (mg/kg/day)	№ Examined	Spermatogenic Endpoints (Mean ± S.D.)		
		Count of Sperm (x10 <sup>6</sup> /g)	Sperm Motility (%)	Sperm Form Anomalies Index (%)
0	20	583.71 ± 149.75	92.24 ± 21.69	7.25 ± 19.94
30	20	632.62 ± 128.30	97.22 ± 2.04	3.97 ± 7.76
100	20	615.37 ± 120.48	97.86 ± 2.07	2.40 ± 2.30
300	19	559.60 ± 184.64	87.62 ± 29.78	10.04 ± 9.02

*Effect on early embryonic development:*

- A significant decrease in the number of corpora lutea as compared with the control group was observed in the 100 mg/kg group. This change was not dose-related and therefore does not appear to be biologically relevant. There were no significant differences between the control and methyl salicylate groups in the numbers of implants or live embryos, pre-implant loss index or dead embryo index.

Dose (mg/kg)	# Pregnant/Total (%)	(Total №) MEAN (± S.D.)		№ (% of the corpora lutea)
		Corpora Lutea	Implantation Site	Pre-implantation loss
0	20/20 (100%)	312 (15.60 ± 1.60)	297 (14.85 ± 1.57)	15 (4.81%)
30	18/20 (90%)	264 (14.67 ± 2.93)	254 (14.11 ± 2.93)	10 (3.79%)
100	18/20 (94.74%)	272 (14.32 ± 1.49)*	267 (14.05 ± 1.54)	5 (1.84%)
300	17/19 (89.5%)	277 (14.58 ± 1.54)	268 (14.11 ± 1.52)	9 (3.25%)

\*: Significantly different from control group (p<0.05)

**Effects of methyl salicylate on litter (i.e., number of viable ad embryos).**

Dose (mg/kg)	№ of Viable Embryos (Mean ± S.D.)	№ of Dead Embryos (% of the # implants)
0	279 (13.95 ± 1.70)	18 (4.81)
30	239 (13.28 ± 2.82)	15 (5.91)
100	253 (13.32 ± 1.77)	14 (5.24)
300	260 (13.68 ± 1.57)	8 (2.99)

**Plasmatic concentration of salicylic acid**

The plasma salicylic acid concentration at 4 hours after administration on day 0 of administration in the 30, 100 and 300 mg/kg groups were 46.4, 147 and 239 µg/ml for the males and 53.5, 164 and 277µg/ml for the females, respectively. The plasma salicylic acid concentration at 4 hours after administration on day 13 of administration in the 30, 100 and 300 mg/kg groups were 46.1, 126 and 290 µg/ml for the males and 47.7, 144 and 300 µg/ml for the females, respectively. These results showed a dose-dependent increase of plasma concentration without clear differences between males and females.

**3.2.1.2 NTP (1984a)**

*Study reference:*

National Toxicology Program. 1984. Methyl salicylate: Reproduction and fertility assessment in CD-1 mice when administered by gavage. NTP, NIEHS report NTP 84-156 (PB 84-241140)

Chapin RE and Sloane RA. Reproductive assessment by continuous breeding: Evolving study design and summaries of ninety studies. Environ Health Perspect. 1997 Feb;105 Suppl 1:199-205.

Morrissey RE, Lamb JC 4th, Morris RW, Chapin RE, Gulati DK, Heindel JJ. Results and evaluations of 48 continuous breeding reproduction studies conducted in mice. Fundam Appl Toxicol. 1989 Nov;13(4):747-77.

## CLH REPORT FOR HEXYL SALICYLATE

Lamb J, Gulati D, Choudhury H, Chambers R, Sabharwal. Methyl salicylate. Environmental Health Perspectives. 1997; 105 Suppl 1.

The description below is taken from Annex I to the CLH Report of methyl salicylate.

### *Detailed study summary and results:*

#### *Test type*

RACB (continuous breeding reproduction study) protocol: task 2 (continuous cohabitation phase) & task 4 (offspring assessment – performed because the overall response in task 2 was negative)

#### *Test substance*

- Methyl salicylate

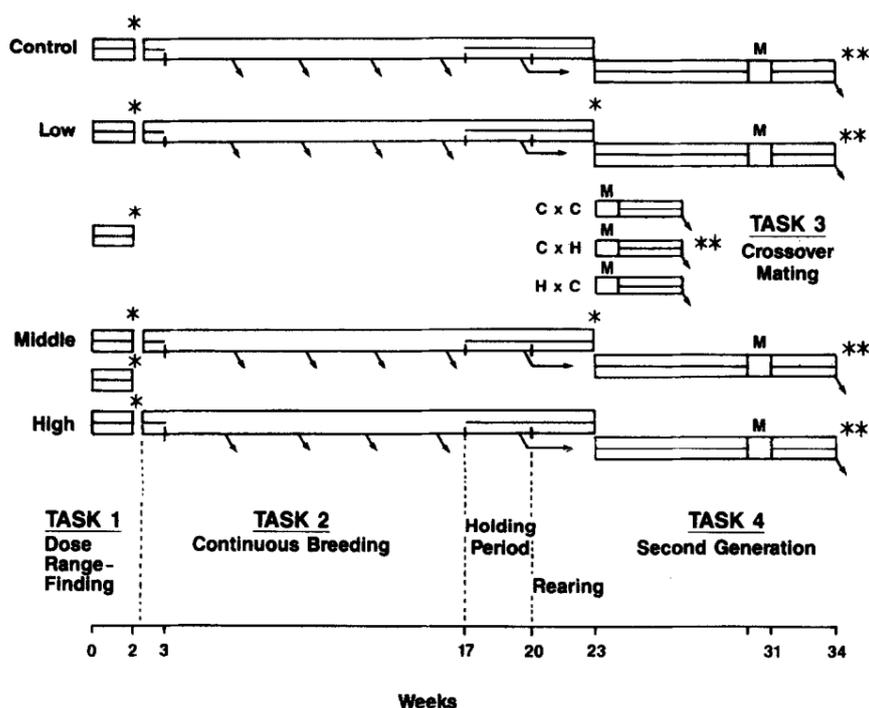
#### *Test animals*

- Swiss CD-1 mice
- n = 40/sex for control and n = 20/sex for treatment groups
- 6 weeks of age

#### *Administration/exposure*

- Oral; gavage
- 0, 25, 50, and 100 mg/kg/day based on food and water consumption, clinical signs and body weights from the Task 1 dose-range-finding study (14 days)
- Vehicle: corn oil
- Chemical was evaluated for purity and for stability in the dosing vehicle for up to 14 days. Any impurity which equalled 1.6% or more of the product was identified.

#### *Description of test design:*



- Task 2: the mice were exposed to the chemical for a 7-day premating period and were then randomly grouped as mating pairs and cohabited and treated continuously for 98 days. Data were collected on all newborns during this period (body weight, proportion of males, number of litters per pair, number of live and dead pups) within 12 hour of birth, after which each litter was discarded. After the 98-day cohabitation, the pairs were separated but continued on treatment. During the next 21 days, any final litters were delivered and kept for at least 21 days (weaning).
- Task 4: the last litter in Task 2 from the control and high dose groups was reared by the dams until weaning (postnatal day 21) and then dosed with methyl salicylate until the mating at approximately postnatal day 74. For this, male offspring were mated to female offspring from the same treatment group and the F2 litters were examined for litter size, sex and pup weight.
- Parameters assessed: see figure below.

### ***Results and discussion***

- The Cochran-Armitage test was used to test for a dose-related trend in fertility. Pairwise comparisons involving mating and fertility indices were performed using Fischer's exact test. Dose group means for number of litters, number of live pups per litter, proportion of live pups, sex ratio were tested for ordered differences using Jonckheere's test. Pairwise comparisons of treatment group means were performed by applying the Wilcoxon-Mann-Whitney U test. A Kruskal-Wallis was also performed on average pup weight. Since the number of pups in a litter may affect the average weight of the litter, an analysis of covariance was also used to test for treatment differences in average pup weight, adjusting for average litter size (live and dead pups). Pairwise comparisons were done using a two-sided t test. For the organ weights, least-squares treatment group means were generated from an analysis of covariance (with body weight as the covariate) and were tested for overall equality using the F test and for pairwise equality using a t test. All comparisons were two-sided. The Kruskal-Wallis and Wilcoxon-Mann-Whitney U tests were also employed. Historical control data were analysed and statistical sensitivity was calculated.
- There was no adverse effect of methyl salicylate exposure on the reproductive and developmental endpoints measured.

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F <sub>0</sub> generation	Dose concentration →	25 mg/kg	50 mg/kg	100 mg/kg
General toxicity		Male, female	Male, female	Male, female
Body weight		—, —	—, —	—, —
Kidney weight <sup>a</sup>		•	•	•
Liver weight <sup>a</sup>		•	•	•
Mortality		—	—	—
Feed consumption		•	•	•
Water consumption		•	•	•
Clinical signs		—	—	—
<b>Reproductive toxicity</b>				
$\bar{x}$ litters/pair		—	—	—
# live pups/litter; pup wt./litter		—, —	—, —	—, —
Cumulative days to litter		—	—	—
Absolute testis, epididymis weight <sup>a</sup>		•	•	•
Sex accessory gland weight <sup>a</sup> (prostate, seminal vesicle)		•	•	•
Epidid. sperm parameters (#, motility, morphology)		•	•	•
Estrous cycle length		•	•	•
<b>Determination of affected sex (crossover)</b>				
Dose level		Male	Female	Both
		•	•	•
F <sub>1</sub> generation	Dose concentration →	•	•	100 mg/kg
General toxicity		Male, female	Male, female	Male, female
Pup growth to weaning		•	•	—, —
Mortality		•	•	—, —
Adult body weight		•	•	—, —
Kidney weight <sup>a</sup>		•	•	•
Liver weight <sup>a</sup>		•	•	—, —
Feed consumption		•	•	•
Water consumption		•	•	•
Clinical signs		•	•	—, —
<b>Reproductive toxicity</b>				
Fertility index		—	—	—
# live pups/litter; pup wt./litter		—, —	—, —	—, —
Absolute testis, epididymis weight <sup>a</sup>		•	•	—, —
Sex accessory gland weight <sup>a</sup> (prostate, seminal vesicle)		•	•	—, —
Epidid. sperm parameters (#, motility, morphology)		•	•	—, —, —
Estrous cycle length		•	•	•
<b>Summary information</b>				
Affected sex?	Unclear			
Study confounders:	None			
F <sub>1</sub> more sensitive than F <sub>0</sub> ?	No			
Postnatal toxicity:	No			

Legend: —, no change; •, no observation; ↑ or ↓, statistically significant change (p<0.05); —, —, no change in males or females. <sup>a</sup>Adjusted for body weight.

### 3.2.1.3 NTP (1984b)

#### Study reference:

National Toxicology Program. Methyl salicylate: Reproduction and fertility assessment in CD-1 mice when administered by gavage. NTP, NIEHS Report No. NTP-85-022, November 1984 (PB85-164283)

Chapin RE and Sloane RA. 1997. Reproductive assessment by continuous breeding: Evolving study design and summaries of ninety studies. Environ Health Perspect.;105 (1):199-205.

Morrissey RE, Lamb JC 4th, Morris RW, Chapin RE, Gulati DK, Heindel JJ. 1989. Results and evaluations of 48 continuous breeding reproduction studies conducted in mice. Fundam Appl Toxicol.;13(4):747-77.

The description below is taken from Annex I to the CLH Report of methyl salicylate.

#### Detailed study summary and results:

### ***Test type***

FACB (fertility assessment by continuous breeding) protocol: task 2 (continuous cohabitation phase) & task 3 (crossover mating – performed if the fertility in the task 2 is significantly affected)

GLP

### ***Test substance***

- Methyl salicylate
- Purity  $\geq$  99%
- Lot 703535

### ***Test animals***

- (COBS) CD-1, (ICR)BR outbred albino mice
- 40 animals/sex in the control group and 20 animals/sex in the treated groups.

### ***Administration/exposure***

- Oral, gavage
- 0, 100, 250, 500 mg/kg bw/day based on the Task 1 (14-day preliminary study). In the task 1, mice received 0, 50, 100, 250, 500 or 1000 mg/kg bw/day for 2 weeks. There was no discernible effect on body weight. Seven animals died during the study: 2 in the control group, 2 in the 50 mg/kg bw/day group and 3 at the highest dose.
- Corn oil at 10 ml/kg bw
- Dosing solutions were prepared every 2 weeks. Aliquots of various dosage formulations were sent for chemical analysis: they were within 93 and 102 % of the indicated methyl salicylate concentrations. These limits were considered acceptable.
- The actual amounts of methyl salicylate and the volume of corn oil gavaged were based on the body weight at the beginning of each week.

### ***Description of test design:***

- Task 2: Male and female mice were exposed to the chemical during 7-day pre-mating period, after which they were randomly paired (1 male : 1 female) within each dose group. Cohabitation was continued for 100 days. Newborn litters were evaluated and immediately sacrificed. Parameters assessed: mortality, body weight, body weight gain, clinical signs, fertility index, litter per pair, live pup per litter, proportion of pups born alive, sex of pups born alive, live pup weight.
- Task 3: animals from the 500 mg/kg bw/day group were tested in a crossover mating trial to determine whether the males or females or both sexes had compromised reproductive performance when matched with control animals. Animals did not receive any treatment between days 127 (week 19) and day 155 (week 23) of the study. Parameters assessed: mating index, fertility index, live pup per litter, proportion of pups born alive, sex of pups born alive, live pup weight.

### ***Results and discussion***

#### **TASK 2**

- Eleven animals died during Task 2; 3 in the control, 2 each in the 100 and 250 mg/kg dose groups and 4 in the 500 mg/kg dose group. The cause varied from case to case but was neither chemical nor dose related. No distinct treatment related symptoms of toxicity were observed during routine health surveillance
- Methyl salicylate had no apparent effect on male or female body weights.

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Treatment Group (g/kg)	Number of Animals			Percent Mortality d(0-14)	Body Weight ± SE		Percent Change in Body Weight d(0-14)
	d=0	d=14			d=0	d=14	
Control	M	8	7	12.5 <sup>a</sup>	35.6 ± 0.76	36.1 ± 0.67	+1.4
	F	8	7	12.5 <sup>a</sup>	26.3 ± 0.66	26.7 ± 0.48	+1.5
0.05	M	8	8	0	35.2 ± 0.53	34.5 ± 0.66	-2.0
	F	8	6	25.0 <sup>b</sup>	27.0 ± 0.48	27.1 ± 0.50	+0.4
0.10	M	8	8	0	35.4 ± 0.62	35.0 ± 0.56	-1.1
	F	8	8	0	26.7 ± 0.50	26.3 ± 0.36	-1.5
0.25	M	8	8	0	35.3 ± 0.67	34.9 ± 0.76	-1.1
	F	8	8	0	27.1 ± 0.34	25.7 ± 0.60	-5.2
0.50	M	8	8	0	34.5 ± 0.58	34.9 ± 0.72	+1.2
	F	8	8	0	27.6 ± 0.60	27.6 ± 0.65	0.0
1.00	M	8	6	25.0 <sup>a</sup>	35.6 ± 0.52	35.1 ± 1.04	-1.4
	F	8	7	12.5 <sup>b</sup>	26.4 ± 0.47	27.9 ± 0.65	+5.7

- The fertility index in the control and various treatment groups varied between 94 to 100%; all breeding pairs except 1 in the 100 mg/kg group delivered at least one litter. Data from breeding pairs in which one or both animals died during task 2 were excluded when computing the average number of litters pair pair, live pups per litter, proportion of pups born alive, sex ratio and live pup weight.

Table 3. Fertility of Pairs During Continuous Breeding (Task 2) Methyl Salicylate

Treatment Group	No. Fertile/ No. Cohabited	Fertility Index (%) a, b
Control	38/38	100
0.1 g/kg	17/18	94
0.25 g/kg	18/18	100
0.50 g/kg	16/16	100

a) Fertility Index (%) =  $\frac{\text{No. Fertile}}{\text{No. Cohabite}} \times 100$

b) Data from breeding pairs in which one partner died during cohabitation were excluded.

- There was a significant decrease (p<0.05) in the mean number of litters at the highest dose. The average number of pups per litter, the proportion of pups born alive, and mean live pup weight values were also significantly reduced (p<0.05) in the 500 mg/kg group compared to the corresponding controls.

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Table 4. Reproductive Performance of Fertile Pairs During Continuous Breeding (Task 2) Methyl Salicylate

Reproductive <sup>a</sup> Parameter	Treatment Group (Methyl Salicylate concentration)			
	Control	0.10 g/kg MS <sup>c</sup>	0.25 g/kg MS	0.5 g/kg MS
LITTERS PER PAIR	4.92 ± 0.044(30) <sup>d</sup>	4.82 ± 0.128(17)	4.78 ± 0.129(18)	4.50 ± 0.258(16) <sup>e</sup>
LIVE PUPS PER LITTER				
Male	5.73 ± 0.209(30)	5.41 ± 0.249(17)	4.75 ± 0.384(18)	4.32 ± 0.364(16) <sup>f, g</sup>
Female	5.55 ± 0.206(30)	5.05 ± 0.228(17)	4.83 ± 0.313(18)	3.46 ± 0.351(16) <sup>f, h, i</sup>
Combined	11.29 ± 0.364(30)	10.47 ± 0.348(17)	9.58 ± 0.648(18)	7.78 ± 0.635(16) <sup>f, h</sup>
PROPORTION OF PUPS BORN ALIVE	0.97 ± 0.010(38)	0.98 ± 0.010(17)	0.96 ± 0.017(18)	0.91 ± 0.033(16) <sup>e, g</sup>
SEX OF PUPS BORN ALIVE (MALES/TOTAL)	0.51 ± 0.010(38)	0.52 ± 0.016(17)	0.49 ± 0.014(18)	0.56 ± 0.024(16) <sup>i</sup>
LIVE PUP WEIGHT (g)				
Male	1.65 ± 0.014(38)	1.64 ± 0.020(17)	1.64 ± 0.018(18)	1.60 ± 0.023(16)
Female	1.60 ± 0.014(38)	1.59 ± 0.021(17)	1.57 ± 0.020(18) <sup>e</sup>	1.53 ± 0.016(16) <sup>e, g</sup>
Combined	1.62 ± 0.013(38)	1.62 ± 0.021(17)	1.60 ± 0.017(18)	1.57 ± 0.021(16) <sup>e</sup>
ADJUSTED LIVE PUP WEIGHT (g) <sup>b</sup>				
Male	1.67 ± 0.014(38)	1.65 ± 0.019(17)	1.63 ± 0.019(18)	1.57 ± 0.021(16) <sup>f, h, i</sup>
Female	1.61 ± 0.013(38)	1.59 ± 0.019(17)	1.56 ± 0.018(18) <sup>e</sup>	1.51 ± 0.021(16) <sup>f, h</sup>
Combined	1.64 ± 0.013(38)	1.62 ± 0.018(17)	1.59 ± 0.018(18) <sup>e</sup>	1.54 ± 0.020(16) <sup>f, h</sup>

a: Mean ± SE.  
b: Means adjusted for total number of live and dead pups per litter by analysis of covariance.  
c: MS = Methyl Salicylate.  
d: Number of pairs providing data indicated in parenthesis.  
e: Significantly different (p<0.05) from the 0 g/kg group.  
f: Significantly different (p<0.01) from the 0 g/kg group.  
g: Significantly different (p<0.05) from the 0.1 g/kg group.  
h: Significantly different (p<0.01) from the 0.1 g/kg group.  
i: Significantly different (p<0.05) from the 0.25 g/kg group.

## TASK 3

- First trial: both the fertility and mating index values in the control were considerably lower than expected. Only 5 out of 17 breeding pairs delivered pups in the control group. These data were considered unacceptable and Task 3 was repeated.

Table 5. Mating and Fertility of Pairs After a Mating Trial to Determine the Affected Sex in the 0.5g/kg Dose Group (Task 3 - 1st Trial) Methyl Salicylate

Treatment Group	No. with Copulatory Plugs/ No. Cohabited	Mating Index (%) <sup>a</sup>	No. Fertile/ No. Cohabited	Fertility Index (%) <sup>b</sup>
Control Male vs. Control Female	4/17 <sup>c</sup>	24 <sup>d</sup>	5/17	29
0.5 g/kg Male vs. Control Female	11/18	61	13/18	72 <sup>e</sup>
Control Male vs. 0.5 g/kg Female	8/18	44	8/18	44

a: Mating Index (%) =  $\frac{\text{No. with Copulatory Plugs}}{\text{No. Cohabited}} \times 100$

b: Fertility Index (%) =  $\frac{\text{No. Fertile}}{\text{No. Cohabited}} \times 100$

c: One female animal died the day after the initiation of mating; therefore, the total number of breeding pairs was reduced from 18 to 17.  
d: Due to certain unknown reasons, both fertility and mating index values were considerably lower than expected. Task 3 was repeated. See data in Tables 7 and 8.  
e: Significantly different (p<0.01) from the control male vs. control female group.

- Second trial: fertility in all three groups, including control, was poor and essentially the same. It was still not possible to determine which sex was affected by the treatment.

Table 7. Mating and Fertility of Pairs After a Mating Trial to Determine the Affected Sex in the 0.5 g/kg Dose Group (Task 3 - 2nd Trial) Methyl Salicylate

Treatment Group	No. with Copulatory Plugs /No. Cohabited	Mating Index (%) <sup>a</sup>	No. Fertile/ No. Cohabited	Fertility Index (%) <sup>b</sup>
Control Male vs. Control Female	10/17	59	7/17	41
0.5 g/kg Male vs. Control Female	7/18	39	8/18	44
Control Male vs. 0.5 g/kg Female	10/18	56	11/18	61

a: Mating Index (%) =  $\frac{\text{No. with Copulatory Plugs}}{\text{No. Cohabited}} \times 100$

b: Fertility Index (%) =  $\frac{\text{No. Fertile}}{\text{No. Cohabited}} \times 100$

Table 8. Reproductive Performance of Fertile Pairs to Determine the Affected Sex in the 0.5 g/kg Dose Group (Task 3 - 2nd Trial) Methyl Salicylate

Reproductive Parameter	Treatment Group		
	Control Male vs. Control Female	0.5 g/kg MS Male vs. Control Female <sup>c</sup>	Control Male vs. 0.5 g/kg MS Female
LIVE PUPS PER LITTER			
Male	2.57 ± 0.75(7) <sup>a,b</sup>	2.63 ± 0.63(8)	2.18 ± 0.33(11)
Female	3.71 ± 0.71(7)	2.75 ± 0.77(8)	3.09 ± 0.83(11)
Combined	6.29 ± 1.27(7)	5.37 ± 0.94(8)	5.18 ± 0.89(11)
PROPORTION OF PUPS BORN ALIVE	0.87 ± 0.08(7)	0.89 ± 0.10(8)	0.89 ± 0.06(11)
SEX OF PUPS BORN ALIVE (MALES/TOTAL)	0.42 ± 0.12(7)	0.52 ± 0.12(8)	0.52 ± 0.12(11)
LIVE PUP WEIGHT (g)			
Male	1.81 ± 0.09(6)	1.79 ± 0.09(7)	1.79 ± 0.03(10)
Female	1.68 ± 0.05(6)	1.81 ± 0.05(7)	1.69 ± 0.04(10)
Combined	1.74 ± 0.06(7)	1.83 ± 0.07(8)	1.83 ± 0.10(11)
ADJUSTED LIVE PUP WEIGHT (g) <sup>d</sup>			
Male	1.83 ± 0.07(6)	1.80 ± 0.06(7)	1.77 ± 0.05(10)
Female	1.70 ± 0.05(6)	1.81 ± 0.05(7)	1.68 ± 0.04(10)
Combined	1.76 ± 0.09(7)	1.83 ± 0.09(8)	1.81 ± 0.06(11)

a: Mean ± SE.

b: Number of fertile pairs providing data, indicated in parenthesis.

c: MS = Methyl Salicylate.

d: Pup weight adjusted for total number of live and dead pups per litter by analysis of covariance.

### 3.2.1.4 Collins *et al.* (1971)

#### Study reference:

Collins T.F.X, Hansen W.H, Keeler HV. Effect of methyl salicylate on rat reproduction. Toxicology and applied pharmacology 18, 755-765 (1971)

The description below is taken from Annex I to the CLH Report of methyl salicylate.

#### Detailed study summary and results:

##### Test type

3 generation study

##### Test substance

- Methyl salicylate
- Source: Dodge and Olcott, Inc., New York (0302655151)

### ***Test animals***

- Osborne-Mendel rats
- 20/sex/group

### ***Administration/exposure***

- Oral (feed)
- 0, 500, 1500, 3000, 5000 ppm
- The diet was prepared every 14 days

### ***Description of test design:***

- The animals fed methyl salicylate at the respective doses for 100 days, after which the animals were mated.
- Parameters assessed in parents: fertility index (number of litters cast/number of females exposed to mating)
- After the birth of the first litter (F1a) observations were made of the number of stillborn and liveborn young and of grossly visible abnormalities. Litters were similarly observed on day 4 and counts were made of the number and conditions of the living pups. When litters exceeded 10 at day 4, the number of pups were reduced to 10. At weaning the animals were sacrificed. The parents (F0) were remated and the same observations were made on the second litter (F1b). At weaning, 20 littermated pairs were selected at each dose level to produce the next generation. The same procedure was followed for succeeding generations except that animals of the third generation were killed and autopsied.
- Supplemental study: in order to test the efficacy of calcium in alleviating or reversing any adverse effect of methyl salicylate, a separate group of F2b rats from each dose level were fed 1500 ppm calcium carbonate in addition to methyl salicylate. The animals were mated and their first and second litters were observed as previously described. Of the 1500 ppm calcium carbonate fed, approximately 600 ppm was available as calcium.

### ***Results and discussion***

- A 2-sided chi-square test was used to determine significant differences between each dose and control for fertility index and average litter size. No statistical tests were made on the viability, survival or weaning indexes.
- There was no statistically significant difference ( $P < 0.05$ ) for the fertility index at any level. Appreciable decreases can be seen, however, in the second generation matings at the 5000 ppm level.

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TABLE 1  
FERTILITY INDEXES OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

Generation	Mating	Dietary level (ppm)									
		0		500		1500		3000		5000	
		FI <sup>a</sup>	% <sup>b</sup>	FI	%	FI	%	FI	%	FI	%
1	1	20/20	100	20/20	100	20/20	100	20/20	100	20/20	100
	2	19/19	100	20/20	100	18/19	95	19/19	100	20/20	100
2	1	20/20	100	19/20	95	20/20	100	19/20	95	17/20	85
	2	19/19	100	19/20	95	19/19	100	19/20	95	10/13	77
3	1	20/20	100	18/20	90	18/19	95	19/20	95	17/19	89
	2	18/20	90	16/18	89	17/19	89	15/17	88	16/19	84
1, 2, 3 <sup>c</sup>	1	60/60	100	57/60	95	58/59	98	58/60	97	54/59	92
	2	56/58	97	55/58	95	54/57	95	53/56	95	46/52	88
3(+Ca) <sup>d</sup>	1	20/20	100	19/20	95	19/20	95	17/20	85	5/5	100
	2	19/20	95	16/20	80	16/20	80	13/20	65	5/5	100

<sup>a</sup> Fertility index (number of litters cast/number of females exposed to mating).

<sup>b</sup> Percent females pregnant.

<sup>c</sup> Not analyzed for statistical significance.

<sup>d</sup> Calcium carbonate (1500 ppm) added to diet.

<sup>3</sup> Source of calcium carbonate: Fisher Scientific, Fairlawn, New Jersey (763830).

- Significant decreases in average litter size was found in the second generation in the second mating at 3000 ppm ( $P < 0.05$ ) and in both matings at 5000 ppm ( $P < 0.01$ ). Although decreases were seen at 1500 ppm, they were not statistically significant because of the large variation in progeny between females within a group. A dose-related decrease in average litter size per female exposed was apparent in both matings of the second generation starting at 1500 ppm. When the results were combined for all generations, mating 1 and mating 2, there was a clear dose-related decrease starting at 1500 ppm. When the animals were fed methyl salicylate plus calcium carbonate, significant decreases appeared in the first mating at 3000 ppm ( $P < 0.01$ ) and 5000 ppm ( $P < 0.05$ ).

TABLE 2  
AVERAGE LITTER SIZE OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

Generation	Mating	Dietary level (ppm)									
		0		500		1500		3000		5000	
		No. <sup>a</sup>	Av. <sup>b</sup>	No.	Av.	No.	Av.	No.	Av.	No.	Av.
1	1	208/20	10.4	211/19	11.1	207/20	10.4	235/20	11.8	188/18	10.4
	2	213/19	11.2	232/20	11.6	228/19	12.0	238/19	12.5	198/19	10.4
2	1	216/20	10.8	205/20	10.2	206/20	10.3	169/20	8.4	124/20	6.2 <sup>c</sup>
	2	226/19	11.9	204/20	10.2	189/18	10.5	187/20	9.4 <sup>d</sup>	86/13	6.6 <sup>c</sup>
3	1	192/20	9.6	188/19	9.9	172/19	9.1	170/20	8.5	179/19	9.4
	2	197/20	9.8	191/18	10.6	163/19	8.6	132/17	7.8	172/19	9.1
1, 2, 3 <sup>e</sup>	1	616/60	10.3	604/58	10.4	585/59	9.9	574/60	9.6	491/57	8.6
	2	636/58	11.0	627/58	10.8	580/56	10.4	557/56	9.9	456/51	8.9
3(+Ca) <sup>f</sup>	1	201/20	10.0	188/20	9.4	173/20	8.6	130/20	6.5 <sup>c</sup>	35/5	7.0 <sup>d</sup>
	2	181/20	9.0	179/20	9.0	148/20	7.4	127/20	6.4	43/5	8.6

<sup>a</sup> Total number progeny/number females exposed to mating.

<sup>b</sup> Average litter size per female exposed to mating.

<sup>c</sup> Significant at  $P < 0.01$ .

<sup>d</sup> Significant at  $P < 0.05$ .

<sup>e</sup> Not analyzed for statistical significance.

<sup>f</sup> Calcium carbonate (1500 ppm) added to diet.

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- Statistically significant differences were observed in the average number of progeny both matings of the second generation at 3000 ppm ( $P < 0.05$ ) and at 5000 ppm ( $P < 0.01$ ). As with average litter size, average liveborn progeny for both matings of the second generation show a consistent dose-related decrease. When the data from all generations are combined for mating 1 and mating 2, there is a consistent decrease with increased dose level starting at 1500 ppm. In the animals fed methyl salicylate and calcium, the average number of liveborn progeny per female exposed showed statistically significant decreases in the first mating at the 3 dose levels from 1500 to 5000 ppm and in the second mating at 3000 ppm. Statistically significant decreases of viability, survival and weaning index were noted in the second generation from 3000 ppm.

TABLE 5  
VIABILITY DATA FOR RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

Generation	Mating	Dietary level (ppm)														
		0			500			1500			3000			5000		
		No. <sup>a</sup>	Av. <sup>b</sup>	VI <sup>c,d</sup>	No.	Av.	VI	No.	Av.	VI	No.	Av.	VI	No.	Av.	VI
1	1	208/20	10.4	1.00	211/19	11.1	1.00	195/20	9.8	0.94	229/20	11.4	0.97	167/18	9.3	0.88
	2	213/19	11.2	1.00	231/20	11.6	1.00	226/19	11.9	0.99	237/19	12.5	1.00	189/19	9.9	0.95
2	1	215/20	10.8	1.00	203/20	10.2	0.99	203/20	10.2	0.99	164/20	8.2 <sup>e</sup>	0.97	106/19	5.6 <sup>f</sup>	0.85
	2	225/19	11.8	1.00	203/20	10.2	1.00	189/18	10.5	1.00	182/20	9.1 <sup>e</sup>	0.97	82/13	6.3 <sup>f</sup>	0.95
3	1	188/20	9.4	0.98	184/19	9.7	0.98	160/19	8.4	0.93	164/20	8.2	0.96	174/19	9.2	0.97
	2	196/20	9.8	1.00	186/18	10.3	0.97	155/19	8.2	0.95	118/17	6.9	0.89	166/19	8.7	0.97
1, 2, 3 <sup>d</sup>	1	611/60	10.2	0.99	598/58	10.3	0.99	558/59	9.5	0.95	557/60	9.3	0.97	447/56	8.0	0.91
	2	634/58	10.9	1.00	620/58	10.7	0.99	570/56	10.2	0.98	537/56	9.6	0.96	437/51	8.6	0.96
3(+Ca) <sup>g</sup>	1	199/20	10.0	0.99	186/20	9.3	0.99	160/20	8.0 <sup>e</sup>	0.92	123/20	6.2 <sup>f</sup>	0.95	35/5	7.0 <sup>e</sup>	1.00
	2	176/20	8.8	0.97	175/20	8.8	0.98	146/20	7.3	0.99	126/20	6.3 <sup>e</sup>	0.99	42/5	8.4	0.98

<sup>a</sup> Total number liveborn/number females exposed to mating.

<sup>b</sup> Average number liveborn per female exposed to mating.

<sup>c</sup> Viability index (no. liveborn/total no. born).

<sup>d</sup> Not analyzed for statistical significance.

<sup>e</sup> Significant at  $P < 0.05$ .

<sup>f</sup> Significant at  $P < 0.01$ .

<sup>g</sup> Calcium carbonate (1500 ppm) added to diet.

TABLE 7  
SURVIVAL DATA OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

Generation	Mating	Dietary level (ppm)														
		0			500			1500			3000			5000		
		No. <sup>a</sup>	Av. <sup>b</sup>	SI <sup>c,d</sup>	No.	Av.	SI	No.	Av.	SI	No.	Av.	SI	No.	Av.	SI
1	1	157/17	9.2	0.90	116/14	8.3	0.82	172/19	9.1	0.96	152/15	10.1	0.92	129/15	8.6	0.94
	2	202/19	10.6	0.95	196/20	9.8	0.85	205/19	10.8	0.91	218/19	11.5	0.92	168/19	8.8	0.89
2	1	188/20	9.4	0.87	179/20	9.0	0.88	190/20	9.5	0.94	123/20	6.2 <sup>e</sup>	0.75	82/19	4.3 <sup>f</sup>	0.77
	2	211/19	11.1	0.94	188/20	9.4	0.93	186/18	10.3	0.98	165/20	8.2 <sup>e</sup>	0.91	61/13	4.7 <sup>f</sup>	0.74
3	1	174/20	8.7	0.93	177/19	9.3	0.96	147/19	7.7	0.92	139/20	7.0	0.85	147/19	7.7	0.84
	2	174/20	8.7	0.89	179/18	9.9	0.96	150/19	7.9	0.97	113/17	6.6	0.96	153/19	8.1	0.92
1, 2, 3 <sup>d</sup>	1	519/57	9.1	0.90	472/53	8.9	0.89	509/58	8.8	0.94	414/55	7.5	0.84	358/53	6.8	0.86
	2	587/58	10.1	0.93	563/58	9.7	0.91	541/56	9.7	0.95	496/56	8.9	0.92	382/51	7.5	0.87
3(+Ca) <sup>g</sup>	1	184/20	9.2	0.92	181/20	9.0	0.97	143/20	7.2	0.89	114/20	5.7 <sup>f</sup>	0.93	33/5	6.6	0.94
	2	139/20	7.0	0.79	162/20	8.1	0.92	132/20	6.6	0.90	106/20	5.3	0.84	37/5	7.4	0.88

<sup>a</sup> Total no. day 4 survivors/no. females exposed to mating.

<sup>b</sup> Average no. day 4 survivors per female exposed to mating.

<sup>c</sup> Survival index (no. day 4 survivors/no. liveborn).

<sup>d</sup> Not analyzed for statistical significance.

<sup>e</sup> Significant at  $p < 0.05$ .

<sup>f</sup> Significant at  $p < 0.01$ .

<sup>g</sup> Calcium carbonate (1500 ppm) added to diet.

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WEANING DATA OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

Generation	Mating	Dietary level (ppm)														
		0			500			1500			3000			5000		
		No. <sup>a</sup>	Av. <sup>b</sup>	WI <sup>c,d</sup>	No.	Av.	WI	No.	Av.	WI	No.	Av.	WI	No.	Av.	WI
1	1	154/17	9.1	0.98	114/14	8.1	0.98	172/19	9.1	1.00	151/15	10.1	0.99	129/15	8.6	1.00
	2	183/19	9.6	0.91	187/20	9.4	0.95	203/19	10.7	0.99	191/19	10.1	0.88	164/19	8.6	0.98
2	1	176/20	8.8	0.94	168/20	8.4	0.94	188/20	9.4	0.99	121/20	6.0 <sup>e</sup>	0.98	74/19	3.9 <sup>f</sup>	0.90
	2	200/19	10.5	0.95	173/20	8.6	0.92	179/18	9.9	0.96	160/20	8.0	0.97	48/13	3.7 <sup>f</sup>	0.79
3	1	170/20	8.5	0.98	172/19	9.1	0.97	146/19	7.7	0.99	122/20	6.1	0.88	137/19	7.2	0.93
	2	170/20	8.5	0.97	179/18	9.9	1.00	149/19	7.8	0.99	111/17	6.5	0.98	144/19	7.6	0.94
1, 2, 3 <sup>d</sup>	1	500/57	8.8	0.96	454/53	8.6	0.96	506/58	8.7	0.99	394/55	7.2	0.95	340/53	6.4	0.95
	2	553/58	9.5	0.94	539/58	9.3	0.96	531/56	9.5	0.98	462/56	8.2	0.93	356/51	7.0	0.93
3(+Ca) <sup>g</sup>	1	183/20	9.2	0.99	181/20	9.0	1.00	140/20	7.0 <sup>e</sup>	0.98	108/20	5.4 <sup>f</sup>	0.94	33/5	6.6	1.00
	2	121/20	6.0	0.87	144/20	7.2	0.89	130/20	6.5	0.98	102/20	5.1	0.96	28/5	5.6	0.76

<sup>a</sup> Total no. of adjusted day 21 survivors/no. females exposed. Adjusted day 21 survivors = (no. alive at day 21)/(no. kept at day 4) × no. alive at day 4.

<sup>b</sup> Average no. of adjusted day 21 survivors per female exposed to mating.

<sup>c</sup> Weaning index (no. of adjusted day 21 survivors/no. alive at day 4).

<sup>d</sup> Not analyzed for statistical significance.

<sup>e</sup> Significant at  $p < 0.05$ .

<sup>f</sup> Significant at  $p < 0.01$ .

<sup>g</sup> Calcium carbonate (1500 ppm) added to diet.

- Decreases in weight at weanling appeared consistently from 3000 ppm.

WEANLING WEIGHTS BY SEX OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

Generation	Mating	Dietary level (ppm)									
		0		500		1500		3000		5000	
		M	F	M	F	M	F	M	F	M	F
1	1	40.1 <sup>a</sup>	41.3	40.7	38.8	38.0	37.0	32.9	32.6	34.8	33.9
	2	47.0	45.2	45.6	43.0	45.4	42.6	42.1	41.2	37.1	37.5
2	1	46.9	43.6	40.1	39.4	41.1	40.2	40.2	39.4	37.4	34.9
	2	49.0	47.2	43.4	41.0	43.3	42.4	44.6	40.7	42.5	40.2
3	1	46.2	45.5	43.1	41.9	42.8	40.6	42.7	41.9	34.6	31.5
	2	46.3	42.6	46.2	44.6	47.3	46.0	44.7	41.7	38.6	34.6
3(+Ca) <sup>b</sup>	1	48.0	45.7	43.2	40.6	45.9	40.8	40.9	40.2	42.3	40.7
	2	45.6	44.2	39.4	36.3	39.7	38.2	42.8	40.0	36.8	32.9

<sup>a</sup> Mean body weight (g).

<sup>b</sup> Calcium carbonate (1500 ppm) added to diet.

- External examination of the newborn and weanling rats from all the litters disclosed no grossly visible abnormalities. In autopsy of the third generation weanlings, findings were negative. Histopathologic examinations of the livers and kidneys of third-generation weanling disclosed no indication of toxic effects.

### 3.2.1.5 Anonymous (1978a)

#### Study reference:

Anonymous. 1978. Methyl salicylate: studies of osseous changes in the rat, reproduction in the rat and mouse and liver and kidney effects in the dog.

The description below is taken from Annex I to the CLH Report of methyl salicylate.

### ***Detailed study summary and results:***

#### ***Test type***

2-generation study

#### ***Test substance***

- Methyl salicylate

#### ***Test animals***

- Wistar rats (Manor Farm)
- 25/sex/group
- Approximately 60 days old at start of the test

#### ***Administration/exposure***

- 0.25 and 0.5% in Purina diet. A negative control group was maintained in the same environment as the test animals.
- Due to the volatility of methyl salicylate only enough diet for one week was prepared at any one time and this was stored in tightly closed, metal cannisters.

#### ***Description of test design:***

- The animals were housed individually in plastic cages with wood chips as litter. The parent stock (F0) were maintained on their assigned diets for 60 days prior to mating. Mating was accomplished by placing a male of the same group with a female for a period of 1 week. The F0 stock were mated twice to produce F1a and F1b litters. The F1a were maintained through weaning; approximately 30 days after weaning the F0 stock were remated. Thirty males and 30 females were randomly selected from the F1b litters of each test and control group to serve as the parent stock for the F2a and F2b litters. The diets were fed to all animals, parent and young, throughout the entire test period, from initiation of the F0 stock through to the weaning of the F2b litters.
- During the littering periods the animals were observed hourly, 24 hours a day to record the number of pups per litter, their viability and condition at time of delivery. Records were maintained of total born, liveborn, number alive at 5 days and number weaned at 21 days. Litters containing more than 10 pups at day 5 were reduced to 10 pups by random selection. From these statistics stillborn, viability, lactation and reproduction indices were calculated. If a female died after weaning an initial litter the statistics of the initial litter would not be included in the overall evaluation. Records were maintained as to the total born, liveborn, live at 5 days and weaned at 21 days. Litters were reduced to 10 pups, by random selection at day 5, and day 21 statistics were suitably adjusted for this reduction. The various formulae of the indices used in evaluating the various phases of reproduction per formance were as follows:
  - Stillborn = no. stillborn/total born x 100
  - Viability = no. alive 5 days/no. liveborn x 100
  - Lactation = no. weaned 21 days/no. alive 5 days x 100
  - Reproduction no. weaned 21 days/no. liveborn x 100

#### ***Results and discussion***

- None of the young born in the litters, including the F1 and F2 litters, were observed to have any gross abnormalities. All young surviving to weaning appeared normal in respect to body growth, appearance and behaviour.

- The mating performance of the 0.25% methylsalicylate group was comparable to that of the negative control group and that of the 0.5% methyl salicylate group showed a higher number of unsuccessful matings.

TABLE VIII

RAT MATING PERFORMANCE  
N\* % Females (Pregnancies/Matings)

		2/2	1/2	0/2
<b>First Generation</b>				
Control	25	60.0	32.0	8.0
0.25%	24	70.8	25.0	4.2
0.50%	23	60.9	17.4	21.7
<b>Second Generation</b>				
Control	28	39.3	39.3	21.4
0.25%	30	33.3	36.7	30.0
0.50%	30	33.3	43.3	23.4
<b>First and Second Generations</b>				
Control	53	49.1	35.9	15.0
0.25%	54	50.0	31.5	18.5
0.50%	53	45.3	32.1	22.6

\*Females mated twice

- In actual numbers the litter size for the negative control group was rather consistently larger than the litter sizes of the two levels of methyl salicylate. The 0.5% methyl salicylate group exhibited a higher number of deaths between birth and 5 days than the 0.25% methyl salicylate and negative control groups.

TABLE IX

MEAN LITTER SIZE - BIRTH THROUGH WEANING

	Total Born	Live Born	Not Killed At Birth	Alive 5 Days	Weaned 21 Days
<b>First Generation</b>					
Control	11.53	11.34	11.32	11.16	9.38
0.25%	10.48	10.38	10.33	10.23	8.85
0.50%	11.03	10.63	10.53	9.38	8.09
<b>Second Generation</b>					
Control	9.55	8.94	8.94	8.76	8.02
0.25%	7.10	6.94	6.94	6.81	5.81
0.50%	8.49	8.33	8.33	7.58	6.41
<b>First and Second Generations</b>					
Control	10.61	10.22	10.21	10.04	8.75
0.25%	9.00	8.87	8.85	8.73	7.53
0.50%	9.74	9.46	9.42	8.46	7.24

- The negative control group experienced a larger number of stillborn than did either of the methyl salicylate groups; the figure ran to 6.35% for the 2 matings in the second generation. The viability indices for the 0.25% methyl salicylate group were comparable to those of the negative control group while those of the 0.5% methyl salicylate group were lower. The overall lactation indices were fairly equal between the 2 methyl salicylate groups and the negative control groups. The reproduction indices were just about equal for the 0.25% methyl salicylate group and the negative control group, both on a generation and overall basis. The 0.5% methyl salicylate had somewhat lower reproduction indices.

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TABLE X

REPRODUCTION PERFORMANCE INDICES

	Stillborn	Viability	Lactation	Reproduction
<b>First Generation</b>				
Control	1.60	98.4	84.1	82.7
0.25%	0.95	98.6	86.8	85.5
0.50%	3.68	88.2	86.3	76.2
<b>Second Generation</b>				
Control	6.35	98.0	91.6	89.8
0.25%	2.27	98.1	85.3	83.7
0.50%	1.08	90.9	84.6	76.9
<b>First and Second Generations</b>				
Control	3.57	98.2	87.1	85.6
0.25%	1.41	98.4	86.3	84.9
0.50%	2.84	89.4	85.5	76.5

- The results of this study were analysed statistically by pooling the results of both matings in a given population. From the manner in which the various indices (stillborn, viability, lactation and reproduction) are calculated it was deemed appropriate to make an arc sine transformation so that standard analyses of variance techniques could be used to test hypotheses relative to treatment effects. Using the results of the analyses of variance, t test were run to determine the significance of treatment differences within each generation and for the pooled values of both generations.

TABLE XI

PROBABILITY (P) VALUES - TOTAL BORN/FEMALE \*

	First Generation			Second Generation			Total		
	N	Mean	P	N	Mean	P	N	Mean	P
Control	25	17.52	—	28	11.25	—	53	14.21	—
0.25%	24	17.46	>.90	30	7.33	0.075	54	11.83	0.15
0.50%	23	15.35	0.40	30	4.33	0.35	53	11.94	0.15

\*Twice mated females compared to controls

TABLE XII

PROBABILITY (P) VALUES - LIVE BORN/FEMALE \*

	First Generation			Second Generation			Total		
	N	Mean	P	N	Mean	P	N	Mean	P
Control	25	17.24	—	28	10.54	—	53	13.70	—
0.25%	24	17.29	>0.90	30	7.17	0.10	54	11.67	0.15
0.50%	23	14.78	0.35	30	9.17	0.50	53	11.17	0.25

\*Twice mated females compared to controls

TABLE XIII

PROBABILITY (P) VALUES - TOTAL WEANED/FEMALE \*

	First Generation			Second Generation			Total		
	N	Mean	P	N	Mean	P	N	Mean	P
Control	25	14.26	—	28	9.44	—	53	11.72	—
0.25%	24	14.78	0.80	30	5.97	0.075	54	9.89	0.25
0.50%	23	11.26	0.20	30	7.05	0.20	53	8.87	0.075

\*Twice mated females compared to controls

- Since both of the methyl salicylate groups had a lower total incidence of stillbirths over the two generations, this index was subjected to t testing. None of these three comparisons, either within each generation or for the combined generation, showed treatment differences to be significant at a level of 0.05 or less.

### 3.2.1.6 Anonymous (1978b)

**Study reference:**

Anonymous. 1978. Methyl salicylate: studies of osseous changes in the rat, reproduction in the rat and mouse and liver and kidney effects in the dog.

The description below is taken from Annex I to the CLH Report of methyl salicylate.

**Detailed study summary and results:**

**Test type**

2-generation study

**Test substance**

- Methyl salicylate

**Test animals**

- Mice
- 25/sex/group
- Approximately 60 days old at start of the test

**Administration/exposure**

- 0.25 and 0.5% in Purina diet. A negative control group was maintained in the same environment as the test animals.
- Due to the volatility of methyl salicylate only enough diet for one week was prepared at any one time and this was stored in tightly closed, metal cannisters.

**Description of test design:**

- The animals were housed individually in plastic cages with wood chips as litter. The parent stock (F0) were maintained on their assigned diets for 30 days prior to mating. Mating was accomplished by placing a male of the same group with a female for a period of 1 week. The F0 stock were mated twice to produce F1a and F1b litters. The F1a were maintained through weaning; approximately 30 days after weaning the F0 stock were remated. Thirty males and 30 females were randomly selected from the F1b litters of each test and control group to serve as the parent stock for the F2a and F2b litters. The diets were fed to all animals, parent and young, throughout the entire test period, from initiation of the F0 stock through to the weaning of the F2b litters.

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- During the littering periods the animals were observed hourly, 24 hours a day to record the number of pups per litter, their viability and condition at time of delivery. Records were maintained of total born, liveborn, number alive at 5 days and number weaned at 21 days. Litters containing more than 10 pups at day 5 were reduced to 10 pups by random selection. From these statistics stillborn, viability, lactation and reproduction indices were calculated.

### Results and discussion

- No physical abnormalities were observed in the young of the 0.25% and 0.5% methyl salicylate groups. All young surviving to weaning exhibited normal development in respect to body growth, appearance and behaviour.
- Conception rate was higher for the two groups on methyl salicylate than for the negative control groups. The number of unsuccessful matings for the females of the negative control group was almost double that of the methyl salicylate groups.

TABLE XIV

	N*	MOUSE MATING PERFORMANCE		
		% Females 2/2	(Pregnancies/Matings)	
			1/2	0/2
<b>First Generation</b>				
Control	20	85.0	5.0	10.0
0.25%	22	72.3	27.3	0.0
0.50%	25	72.0	28.0	0.0
<b>Second Generation</b>				
Control	22	9.1	72.7	18.2
0.25%	18	16.7	66.7	16.6
0.50%	16	18.9	68.8	12.4
<b>First and Second Generation</b>				
Control	42	45.2	40.5	14.3
0.25%	40	47.5	45.0	7.5
0.50%	41	51.2	43.9	4.9

\* Females mated twice

- The negative control group although having a slightly larger litter size at birth experienced higher losses during the 21 day weaning period. At weaning the two methyl salicylate groups had equal or larger numbers of pups, on a litter basis, survive.

TABLE XV

	MEAN LITTER SIZE - BIRTH THROUGH WEANING				
	Total Born	Live Born	Not Killed At Birth	Alive 5 Days	Weaned 21 Days
<b>First Generation</b>					
Control	12.51	12.06	12.03	11.23	9.04
0.25%	10.68	10.58	10.53	10.03	9.06
0.50%	10.56	10.30	10.19	9.42	8.96
<b>Second Generation</b>					
Control	9.80	9.50	9.50	8.25	6.09
0.25%	11.50	11.50	11.50	11.20	7.95
0.50%	9.82	9.82	9.71	9.71	8.89
<b>First and Second Generation</b>					
Control	11.53	11.13	11.11	10.15	7.96
0.25%	10.95	10.88	10.84	10.41	8.70
0.50%	10.35	10.17	10.08	10.08	8.94

- The negative control group had a larger percentage of stillbirths than did the 0.25% and 0.5% methyl salicylate groups. The two salicylate groups had viability, lactation and reproduction indices that were comparable to or better than those of the negative control groups.

TABLE XVI

REPRODUCTION PERFORMANCE INDICES

	Stillborn	Viability	Lactation	Reproduction
<b>First Generation</b>				
Control	3.65	93.1	80.5	75.0
0.25%	0.99	94.8	90.3	85.6
0.50%	2.42	91.4	95.2	87.0
<b>Second Generation</b>				
Control	3.06	86.8	73.8	64.1
0.25%	0.0	97.6	70.8	69.1
0.50%	0.0	98.8	91.6	90.5
<b>First and Second Generation</b>				
Control	3.47	91.2	78.5	71.6
0.25%	0.65	95.7	83.6	80.0
0.50%	1.80	93.4	94.1	88.0

- The results of this mouse reproduction study did not indicate any significant treatment or generation effects attributable to the feeding of 0.25% and 0.5% methyl salicylate.

### 3.2.2 Adverse effects on development

#### 3.2.2.1 Tanaka *et al.* (1973a)

**Study reference:**

Tanaka S, Kawashima K, Nakaura S, Nagao S, Kuwamura T, Takanaka A & Omori Y. 1973a. Studies on the teratogenicity of food additives (3): Teratogenic effect of dietary salicylic acid in rats. *J. Food Hyg. Soc.* 14(6): 549-57.

**Detailed study summary and results:**

**Test type**

Equivalent or similar to OECD Guideline 414. Not GLP compliant.

**Test substance**

Salicylic acid

**Test animals**

- Wistar rats (Nihon Rat Co. Ltd., Tokyo)
- 20 females/group

**Administration/exposure**

- 0.06, 0.1, 0.2, 0.4% in the diet (corresponding to 50.7 +/- 0.6, 77.4 +/- 1.0, 165 +/- 2.1, 205.9 +/- 18.9 mg/kg bw/d).
- No vehicle

- Treatment from gestation day 8 to 14; daily

### ***Description of test design:***

- Females of more than 12 weeks of age weighing about 200 g were housed overnight with males. Copulation was confirmed by the presence of a vaginal plug or sperm in the vagina on the following morning and the day of confirmed copulation was designed as day 0 of gestation.
- On day 20 of gestation, 15 of the 20 animals were sacrificed for fetuses examination. After gross observation, fetuses were divided into 2 groups: one was examined for skeletal bone anomalies and the other for internal organ anomalies. The remaining 5 dams in each group were allowed to deliver their offspring. The offspring were weaned on day 21, their weight and growth were recorded every 3 days and general appearance, behavior and survival were examined daily. After 56 days, the offspring were sacrificed and any visceral or skeletal abnormalities were recorded.
- Determination of salicylic acid concentration in fetuses and maternal organs: 5 dams were fed the diet containing 0.2% salicylic acid from gestation day 8 to 14. They were then sacrificed on gestation day 14 and salicylic acid concentration was fluometrically measured.

### ***Results and discussion***

- In the 0.4% dose group: a marked body weight loss was observed in dams at the beginning of salicylic acid exposure, but a gradual increase in body weight was then observed after gestation day 11 (Figure 2). This decrease in body weight at the beginning of exposure was assumed to be due to a decrease in food intake, but no deaths were observed. Uterine and placental weights were significantly lower than controls, but there were no marked differences in the number of corpora lutea or in the rate of nidation in all groups (Table 1). There was 71.2% neonatal mortality in this group. One dam gave birth to six offspring and all died within a day. Litter size, body weight, body length as well as tail length were statistically significantly decreased (Table 2). At 56 days, external anomalies were observed in 29.6% of offspring (Table 3), internal organ anomalies in 13.6% (Table 4) and skeletal anomalies in 46.8% (Table 5). Maternal effects were expressed as temporary body weight loss with toxic symptoms (salivation, piloerection) and the following fetal effects were observed: high fetal mortality (no live fetuses in 9/15 dams examined), high frequency of complex anomalies (cranioschisis, myeloschisis, pseudomacroglossia, oligodactyly, pes varus etc.) and dose-related fetal growth retardation.
- In the 0.2% dose group: fetal effects (fetal anomalies and growth retardation) were observed in the absence of maternal effects. This dose resulted in a maternal serum concentration of about 116 microgram/mL. The body weight, body length and the tail length were statistically significantly decreased (Table 2). Effects observed at 56 days in offspring were 3.8% external anomalies (Table 3), no internal organ anomalies (Table 4) and 14.6% skeletal anomalies (Table 5).
- In the 0.1% and 0.06% dose groups: no maternal or fetal effects.

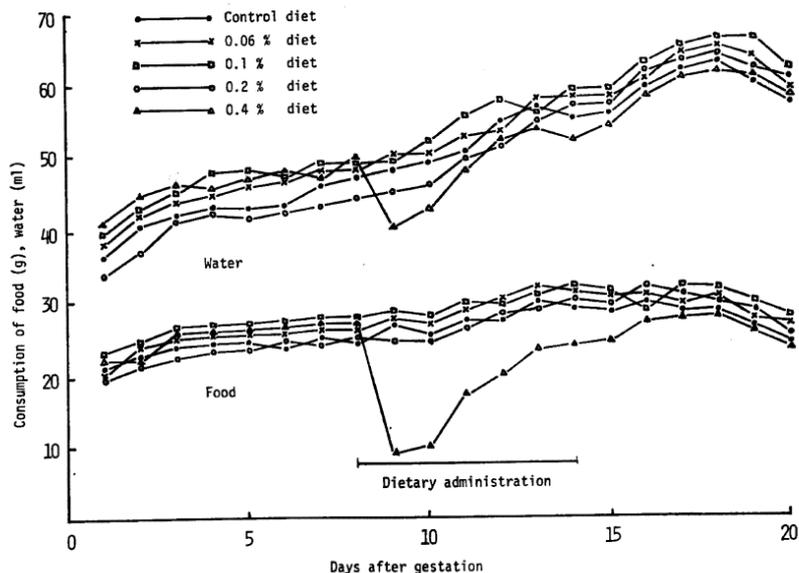


Fig. 2. Effect of dietary salicylic acid on food and water consumptions in pregnant rats

Table 1. Effect of Dietary Salicylic Acid on Pregnant Rats

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
Daily salicylic acid intake (mg/kg) <sup>†</sup>	0	50.7±0.6	77.4±1.0	165.4±2.1	205.9±18.9
No. of dams	15	15	15	15	15
Uterus weight (g) <sup>†</sup>	62.6±4.9	64.4±2.4	67.0±5.0	54.3±3.2	18.0±5.9**
Placental weight (g) <sup>†</sup>	0.44±0.01	0.42±0.01	0.41±0.01*	0.43±0.01	0.36±0.01**
No. of corpora lutea <sup>†</sup>	14.3±0.7	13.9±0.4	14.8±0.7	13.9±0.6	14.2±0.5
No. of implantation <sup>†</sup>	12.3±0.7	12.3±0.4	12.8±1.0	11.2±0.7	12.5±0.9
Rate of nidation (%)	86.1	88.1	86.5	80.4	88.0

<sup>†</sup>: Mean±S.E. \*,\*\* Significant from the control at 5% and 1% level

Table 2. Effect of Dietary Salicylic Acid on Embryonic Development in Rats

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of dams	15	15	15	15	15
Alive fetuses					
No. of animals	172	178	190	160	54
Litter size <sup>†</sup>	11.47±0.96	11.87±0.49	12.71±0.92	10.67±0.65	3.61±1.39**
Body weight (g) <sup>†</sup>	3.46±0.03	3.47±0.02	3.47±0.02	3.11±0.04**	2.24±0.04**
Body length (cm) <sup>†</sup>	3.86±0.01	3.86±0.01	3.87±0.01	3.75±0.02*	3.21±0.03**
Tail length (cm) <sup>†</sup>	1.31±0.01	1.32±0.01	1.26±0.01*	1.29±0.01*	1.14±0.01**
Dead fetuses					
No. of animals	13 (7.0)	6 (3.2)	2 (1.0)	8 (4.8)	134 (71.2)
Resorption	2 (1.1)	1 (0.5)	1 (0.5)	1 (0.6)	45 (23.9)
Placental remnants	11 (5.9)	5 (2.8)	1 (0.5)	7 (4.2)	59 (31.4)
Implantation sites	0	0	0	0	30 (15.9)

<sup>†</sup>: Mean±S.E. \*,\*\* Significant from the control at 5% and 1% level. Numerals in parentheses indicate the ratio (%) to no. of implantations.

Table 3. Incidence of External Anomalies in Fetuses of Rats Given Dietary Salicylic Acid

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of fetuses examined	172	178	190	160	54
No. of fetuses with anomalies	0	0	0	6	16
Occurrence ratio (%)	0	0	0	3.8	29.6
Cranioschisis					4
Pseudomacroglossia					2
Myeloschisis				2	6
Subcutaneous hematocoele				1	
Cracked skin				2	1
Pes varus				1	8
Oligodactyly					6
Syndactyly					3
Flexure of the tail					2
Total	0	0	0	6	32

**Table 4. Incidence of Internal Organ Anomalies in Fetuses of Rats Given Dietary Salicylic Acid**

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of fetuses examined	85	85	97	78	22
No. of fetuses with anomalies	0	0	0	0	3*
Occurrence ratio (%)	0	0	0	0	13.6

\* Anomalies indicate deformed and dislocated kidney and all cases were obtained from one dam in the group.

**Table 5. Incidence of Skeletal Bone Anomalies in Fetuses of Rats Given Dietary Salicylic Acid**

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of fetuses examined	87	98	98	82	32
No. of fetuses with anomalies	0	0	0	12	15
Occurrence ratio (%)	0	0	0	14.6	46.8
Skull					2
Cervical vertebrae				8	12
Thoracic vertebrae				2	4
Rib				2	5
Lumber vertebrae				2	3
Sacral vertebrae					3
Caudal vertebrae				2	1
Radius					5
Total	0	0	0	16	35
No. of fetuses with 14 ribs	6	5	9	54	26
No. of fetuses with 15 ribs	0	0	0	0	3

**Table 6. Effect of Dietary Salicylic Acid During Pregnancy on Offspring of Rats**

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of dams	5	5	4	5	5
Litter size <sup>†</sup>					
at birth	11.0±0.7	11.6±0.5	13.4±1.0	10.2±0.6	6*
3 weeks after birth	10.6±1.5	11.6±0.8	13.8±1.0	9.6±1.8	0
8 weeks after birth	8.4±1.9	8.4±1.4	10.3±2.2	8.2±1.8	0
Weaning rate (%)					
at 3 weeks	96.4	100	100	94.1	—
at 8 weeks	76.4	72.4	78.2	80.4	—

†: Mean±S.E. \*: Youngs were obtained only from 1 dam, but all died within 1 day after birth.

**Table 7. Effect of Dietary Salicylic Acid on Organ Weights of Male Offspring of Rats**

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2
No. of offspring examined	22	10	12	16
Body weight (g)	174.1±7.8	171.9±10.1	222.3±7.4**	187.9±6.8
Body length (cm)	15.2±0.2	15.1±0.3	17.0±0.2**	15.2±0.2
Tail length (cm)	14.4±0.2	13.6±0.3	15.7±0.2**	15.0±0.2
Carcass (g)	122.0±5.5	118.8±6.8	159.5±5.4**	132.0±4.3
Brain (g)	1.64±0.04	1.68±0.02	1.63±0.08	1.65±0.04
Pituitary (mg)	5.96±0.72	4.74±0.96	6.70±0.82	5.78±0.82
Thyroid (mg)	18.91±1.07	16.97±1.33	19.66±1.43	18.72±1.16
Thymus (g)	0.57±0.12	0.54±0.02	0.68±0.07	0.57±0.04
Heart (g)	0.88±0.03	0.89±0.04	1.05±0.04*	0.96±0.04
Lung (g)	2.01±0.14	1.61±1.01	2.22±0.19	1.56±0.08*
Liver (g)	11.43±0.60	11.55±0.76	14.23±0.61	12.01±0.51
Spleen (g)	0.57±0.03	0.72±0.05*	0.63±0.04	0.79±0.03**
Kidney (g)	2.08±0.08	0.14±0.14	2.52±0.09**	2.42±0.11*
Adrenal (mg)	42.46±1.19	42.91±2.01	49.19±2.21*	44.92±1.31
Testis (g)	1.65±0.08	1.58±0.11	1.89±0.05*	1.76±0.07

Figures in the Table represent mean±S.E. \*\*, \* Significant from the control at 5% and 1% level.

**Table 8. Effect of Dietary Salicylic Acid on Organ Weights of Female Offspring of Rats**

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2
No. of offspring examined	19	21	29	25
Body weight (g)	152.4±4.5	156.5±3.7	175.6±2.4**	151.0±5.4
Body length (cm)	14.5±0.2	14.8±0.2	15.7±0.1**	14.5±0.2
Tail length (cm)	13.0±0.2	13.3±0.1	15.1±0.1**	13.7±0.3
Carcass (g)	102.8±3.3	111.7±2.5*	126.0±1.8**	104.0±3.7
Brain (g)	1.61±0.03	1.59±0.04	1.62±0.01	1.53±0.03
Pituitary (mg)	6.60±1.19	5.68±1.12	6.36±0.69	6.04±0.30
Thyroid (mg)	18.41±1.33	19.21±0.77	19.39±0.57	18.74±0.70
Thymus (g)	0.57±0.04	0.57±0.04	0.53±0.02	0.58±0.06
Heart (g)	0.74±0.01	0.78±0.03	0.82±0.03*	0.73±0.02
Lung (g)	1.49±0.11	1.63±0.08	1.55±0.09	1.48±0.06
Liver (g)	9.95±0.52	10.19±0.37	12.57±0.30**	10.51±0.52
Spleen (g)	0.51±0.02	0.55±0.02	0.55±0.02	0.58±0.03
Kidney (g)	1.76±0.06	1.77±0.05	1.81±0.02	1.77±0.06
Adrenal (mg)	46.01±1.55	49.81±1.78	59.25±1.33**	49.37±2.50
Ovary (mg)	71.02±5.01	92.72±4.57*	100.50±3.19**	79.02±4.17
Uterus (g)	0.23±0.02	0.24±0.02	0.27±0.01	0.21±0.01

Figures in the Table represent mean±S.E. \*\*, \* Significant from the control at 5% and 1% level.

**Table 9. Summarized Data on Macroscopic Findings in Offspring of Rats Given Dietary Salicylic Acid**

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
<b>External examination</b>					
No. of offspring examined	55	58	44	51	0
No. of offspring with anomalies	0	0	0	0	—
Occurrence ratio (%)	0	0	0	0	—
<b>Internal organ examination</b>					
No. of offspring examined	42	42	41	41	0
No. of offspring with anomalies	0	0	0	0	—
Occurrence ratio (%)	0	0	0	0	—
<b>Skeletal examination</b>					
No. of offspring examined	28	29	28	29	0
No. of offspring with anomalies	0	0	0	4*	—
Occurrence ratio (%)	0	0	0	13.8	—
No. of offspring with 14 ribs	0	0	0	5	—

\* Anomalies in cervical vertebrae.

### 3.2.2.2 Tanaka *et al.* (1973b)

#### *Study reference:*

Tanaka S, Kawashima K, Nakaura S, Nagao S, Kuwamura T, Takanaka A & Omori Y. 1973b. Studies on teratogenic effects of salicylic acid and aspirin in rats as related to fetal distribution. Department of Pharmacology, National Institute of Hygienic Sciences, Tokyo, Japan. 13 (2): 73-84.

#### *Detailed study summary and results:*

#### *Test type*

Equivalent or similar to OECD Guideline 414. Not GLP compliant.

#### *Test substance*

Salicylic acid

#### *Test animals*

- Wistar rats (Nihon Rat Co. Ltd., Tokyo)
- 20 females/group

#### *Administration/exposure*

- Gavage with 75, 150, 300 mg/kg bw/d in a 0.5% solution of CMC (carboxymethyl cellulose)
- Treatment from gestation day 8 to 14; daily

#### *Description of test design:*

- Females of more than 12 weeks of age weighing about 200 g were housed overnight with males. Copulation was confirmed by the presence of a vaginal plug or sperm in the vagina on the following morning and the day of confirmed copulation was designed as day 0 of gestation.
- The pregnant females were divided into 7 groups. Groups 1, 2 and 3 received 300, 150 and 75 mg/kg salicylic acid. Groups 4, 5 and 6 received the same dose of aspirin. The group 7 received 5 mL/kg of 0.5% solution of CMC as control.
- On day 20 of gestation, 15 of the 20 animals in each group were sacrificed for fetuses examination. After gross observation, fetuses were divided into 2 groups: one was examined for skeletal bone anomalies and the other for internal organ anomalies. The remaining 5 dams in each group were allowed to deliver their offspring. The offspring were weaned on day 21, their weight and growth were recorded every 3 days and general appearance, behavior and survival were examined daily. After 56 days, the offspring were sacrificed and any visceral or skeletal abnormalities were recorded.

- Determination of salicylic acid concentration in fetuses and maternal organs: 5 dams received 150 mg/kg salicylic acid from gestation day 8 to 14. They were then sacrificed on gestation day 14 after the last treatment and salicylic acid concentration was fluometrically measured.

**Results and discussion**

In the 300 mg/kg dose groups, the body weight gains were inhibited with toxic symptoms such as salivation and piloerection, and some animals died within a few days after the beginning of the administration and high fetal mortality prevailed. Decreased uterine weight was observed in females of the 150 and 300 mg/kg dose groups of salicylic acid as compared to controls (Table 1); these groups had 25.7% and 100% fetal mortality, respectively (Table 2).

Table 1 Postmortem findings of pregnant rats treated with oral salicylic acid and aspirin

Treatment Dose (mg/kg)	Control		Salicylic acid			Aspirin		
	0	75	150	300	75	150	300	
No. of dams	15	15	15	12	15	15	14	
Uterus weight (g)†	62.0 ± 1.8	56.0 ± 3.8	43.8 ± 3.1*	1.6 ± 0.2**	57.0 ± 4.2	54.8 ± 2.5	3.9 ± 1.9**	
Placental weight (g)†	0.42 ± 0.01	0.42 ± 0.01	0.37 ± 0.01	0.07 ± 0.01**	0.39 ± 0.01	0.38 ± 0.02	0.13 ± 0.01*	
No. of corpora lutea†	15.1 ± 0.7	14.0 ± 0.4	14.1 ± 0.6	15.0 ± 0.6	15.8 ± 0.8	14.8 ± 0.4	14.9 ± 0.7	
No. of implantations†	12.0 ± 0.4	11.1 ± 0.7	11.9 ± 0.4	11.9 ± 0.4	11.7 ± 0.7	12.6 ± 0.4	11.6 ± 0.6	
Rate of nidation (%)	79.5	79.3	84.4	79.3	74.1	85.1	77.9	

† : Mean ± S.E.    \*, \*\* Significant from the control at 5 % and 1 % level

Table 2 Effects of oral salicylic acid and aspirin on embryonic development in rats

Treatment Dose (mg/kg)	Control		Salicylic acid			Aspirin		
	0	75	150	300	75	150	300	
No. of dams	15	15	15	12	15	15	14	
<b>Live fetuses</b>								
Litter size†	11.5 ± 0.4	10.5 ± 0.7	8.9 ± 0.7*	—	10.9 ± 1.0	11.5 ± 0.5	4††	
Body weight (g)†	3.60 ± 0.02	3.38 ± 0.09	2.82 ± 0.04**	—	3.40 ± 0.03	2.99 ± 0.03**	2.59 ± 0.10**	
Body length (cm)†	3.87 ± 0.01	3.83 ± 0.01	3.55 ± 0.02*	—	3.84 ± 0.02	3.64 ± 0.01	3.33 ± 0.07	
Tail length (cm)†	1.35 ± 0.01	1.35 ± 0.01	1.27 ± 0.01*	—	1.35 ± 0.01	1.30 ± 0.01	1.23 ± 0.05	
<b>Dead fetuses</b>								
No. of animals	7 (3.9)	8 (4.8)	46 (25.7)	142 (100)	12 (6.8)	17 (9.0)	158(97.5)	
Resorptions	0	0	13 ( 7.3)	10 (7.0)	1 (0.6)	6 (3.2)	6 (3.7)	
Placental remnants	7 (3.9)	7 (4.2)	33 (18.4)	46(32.4)	10 (5.7)	11 (5.8)	70(43.2)	
Implantation sites	0	1 (0.6)	0	86(60.6)	1 (0.6)	0	82(50.6)	

† : Mean ± S.E.    \*, \*\* Significant from the control at 5 % and 1 % level. Numerals in parentheses indicate the ratio (%) to implantation. ††They were obtained only from one dam.

In the 150 mg/kg dose group of salicylic acid, litter size and neonatal body weight, body length, and tail length were significantly decreased (Table 2).

The incidences of external, internal, and skeletal anomalies in offspring autopsied at the 56th day were 1.8% (Table 3), 0% (Table 4), and 2.5% (Table 5), respectively, for the 75 mg/kg dose group of salicylic acid and 27.8% (Table 3), 12.7% (Table 4), and 65.7% (Table 5), respectively; for the 150 mg/kg dose group of salicylic acid. The offspring from females of 150 mg/kg dose group of salicylic acid had decreased body length and tail length compared to controls.

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Table 3 Incidence of external anomalies in fetuses of rats treated with oral salicylic acid and aspirin

Treatment Dose (mg/kg)	Control			Salicylic acid			Aspirin		
	0	75	150	300	75	150	300		
No. of fetuses examined	173	158	133	0	164	172	4		
No. of fetuses with anomalies	0	3	37	—	1	22	3		
Occurrence ratio (%)	0	1.8	27.8	—	0.6	12.7	75.0		
Cranioschisis	0	0	10	—	0	2	3		
Open eyelid	0	0	2	—	0	0	2		
Exophthalmos	0	0	0	—	0	0	1		
Pseudomacroglossia	0	0	10	—	0	2	3		
Myeloschisis	0	0	8	—	0	6	0		
Abdominal fissure	0	0	0	—	0	1	0		
Subcutaneous hemorrhage	0	0	4	—	0	3	0		
Cracked skin	0	0	1	—	0	6	0		
Pes varus	0	2	15	—	1	5	1		
Oligodactyly	0	0	5	—	0	1	0		
Polydactyly	0	0	1	—	0	0	0		
Syndactyly	0	0	1	—	0	1	0		
Taillessness	0	1	1	—	0	1	0		
Flexure of the tail	0	0	1	—	0	1	0		
Total	0	3	59	—	1	29	10		

Table 4 Incidence of internal organ anomalies in fetuses of rats treated with oral salicylic acid and aspirin

Treatment Dose (mg/kg)	Control			Salicylic acid			Aspirin		
	0	75	150	300	75	150	300		
No. of fetuses examined	82	78	63	0	77	89	2		
No. of fetuses with anomalies	0	0	8	—	0	5	2		
Occurrence ratio (%)	0	0	12.7	—	0	5.6	100		
Diaphragmatic hernia	0	0	0	—	0	1	0		
Kidney, Absence	0	0	2	—	0	1	0		
Deform	0	0	2	—	0	3	2		
Dislocation	0	0	1	—	0	1	0		
Adrenal, Deform	0	0	1	—	0	0	0		
Ovary, Dislocation	0	0	3	—	0	0	1		
Total	0	0	9	—	0	6	3		

Table 5 Incidence of skeletal anomalies in fetuses of rats treated with oral salicylic acid and aspirin

Treatment Dose (mg/kg)	Control			Salicylic acid			Aspirin		
	0	75	150	300	75	150	300		
No. of fetuses examined	91	80	70	0	87	83	2		
No. of fetuses with anomalies	0	2	46	—	0	26	2		
Occurrence ratio (%)	0	2.5	65.7	—	0	32.5	100		
Skull	0	0	8	—	0	2	2		
Cervical vertebrae	0	1	38	—	0	23	2		
Thoracic vertebrae	0	0	7	—	0	7	2		
Ribs	0	0	9	—	0	6	2		
Lumber vertebrae	0	0	5	—	0	4	1		
Sacral vertebrae	0	1	5	—	0	4	1		
Caudal vertebrae	0	1	10	—	0	0	0		
Radius	0	0	8	—	0	4	0		
Total	0	3	90	—	0	50	10		
No. of fetuses with 14 ribs	6	17	58	—	15	74	1		
No. of fetuses with 15 ribs	0	0	8	—	0	6	1		

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Table 6 Effects of oral salicylic acid and aspirin on organ weights of male offspring of rats

Treatment Dose (mg/kg)	Control		Salicylic acid		Aspirin	
	0	75	75	150	75	150
No. of offspring examined	23	23	11	17	12	
Body weight (g)	185.7 ± 9.3	191.2 ± 5.8	164.3 ± 6.6	193.7 ± 14.9	161.8 ± 8.4	
Body length (cm)	15.20 ± 0.21	15.68 ± 0.14	14.30 ± 0.38*	15.33 ± 0.43	14.79 ± 0.31	
Tail length (cm)	14.96 ± 0.20	15.00 ± 0.12	14.04 ± 0.30*	15.29 ± 0.39	13.73 ± 0.46*	
Carcass (g)	126.5 ± 6.1	141.1 ± 4.4	111.7 ± 4.8	137.8 ± 9.8	112.2 ± 6.6	
Brain (g)	1.56 ± 0.04	1.56 ± 0.08	1.47 ± 0.03	1.49 ± 0.05	1.48 ± 0.06	
Pituitary (mg)	5.93 ± 0.58	7.31 ± 0.47	4.30 ± 0.39	5.38 ± 0.66	5.29 ± 1.16	
Thyroid (mg)	14.84 ± 0.59	18.62 ± 0.84**	14.28 ± 1.06	15.86 ± 1.06	14.65 ± 0.76	
Thymus (g)	0.61 ± 0.09	0.71 ± 0.04	0.62 ± 0.13	0.57 ± 0.12	0.49 ± 0.03*	
Heart (g)	0.89 ± 0.04	0.96 ± 0.04	0.84 ± 0.03	0.99 ± 0.07	0.77 ± 0.04	
Lung (g)	1.40 ± 0.06	1.48 ± 0.06	1.24 ± 0.06	1.87 ± 0.18*	1.39 ± 0.10	
Liver (g)	11.58 ± 0.74	11.33 ± 0.56	9.52 ± 0.31	11.73 ± 1.19	9.45 ± 0.59*	
Spleen (g)	0.59 ± 0.05	0.62 ± 0.03	0.59 ± 0.04	0.58 ± 0.05	0.52 ± 0.03	
Kidney (g)	2.12 ± 0.10	2.08 ± 0.08	1.91 ± 0.08	2.34 ± 0.16	1.83 ± 0.11	
Adrenal (mg)	45.72 ± 1.32	48.87 ± 1.73	35.58 ± 1.37**	43.22 ± 2.86	40.85 ± 2.94	
Testis (g)	1.69 ± 0.07	1.73 ± 0.05	1.61 ± 0.06	1.81 ± 0.10	1.45 ± 0.12	

Figures in the table represent mean ± S.E. \*,\*\* Significant from the control at 5% and 1% level

The thyroid weight of male offspring from the 75 mg/kg dose group of salicylic acid was significantly decreased compared to controls (Table 6). The incidences of external organ, internal organ, and skeletal anomalies in offspring were 0%, 5.0% and 0% respectively, for the 75 mg/kg dose group and 13.7%, 17.2% and 79.2% respectively, for the 150 mg/kg dose group (Table 7, 8 and 9).

Table 7 Incidence of external anomalies in offspring of rats treated with oral salicylic acid and aspirin

Treatment Dose (mg/kg)	Control		Salicylic acid		Aspirin	
	0	75	150	75	150	
No. of offspring examined	56	53	51	54	53	
No. of offspring with anomalies	0	0	7	0	1	
Ratio (%)	—	—	13.7	—	1.9	
Closed eyelid			2			
Microphthalmos				1		
Cranioschisis				1		
Upper median cleft lip				1		
Pseudomacroglossia				1		
Pes varus			2			
Syndactyly			2			
Closed vaginal orifice			1			
Total	0	0	7	0	4	

Table 8 Incidence of internal organ anomalies in offspring of rats treated with oral salicylic acid and aspirin

Treatment Dose (mg/kg)	Control		Salicylic acid		Aspirin	
	0	75	150	75	150	
No. of offspring examined	42	40	29	31	30	
No. of offspring with anomalies	0	2	5	2	5	
Ratio (%)	—	5.0	17.2	6.5	16.7	
Hydronephrosis		1	2	2	4	
Kidney, Deform			1		1	
Ovary, Absence			1			
Uterine horn, Absence			1			
Uterine horn, Retardation			2		1	
Hydrouterus		1				
Total	0	2	7	2	6	

Table 9 Incidence of skeletal anomalies in offspring of rats treated with oral salicylic acid and aspirin

Treatment Dose (mg/kg)	Control		Salicylic acid		Aspirin	
	0	75	150	75	150	
No. of offspring examined	29	26	24	22	25	
No. of offspring with anomalies	0	0	19	0	16	
Ratio (%)	0	0	79.2	0	64.0	
Cervical vertebrae			16		8	
Thoracic vertebrae			4		1	
Sternum vertebrae			5		4	
Ribs			12		8	
Radius			1			
Total	0	0	38	0	21	
14 ribs	2	3	6	1	9	

### 3.2.2.3 Koshakji and Schulert (1973)

#### *Study reference:*

Koshakji RP and Schulert AR. 1973. Biochemical mechanisms of salicylate teratology in the rat. *Biochem. Pharmacol.* 22: 407-416.

#### *Detailed study summary and results:*

##### *Test type*

No guideline followed. Not GLP compliant.

##### *Test substance*

Salicylic acid

##### *Test animals*

- Sprague-Dawley rats
- 17 females

##### *Administration/exposure*

- Subcutaneous exposure to 380 mg/kg salicylic acid (nominal concentration)
- Vehicle: water

##### *Description of test design:*

- 100 days old females weighing 190-220 g were housed overnight with males. Copulation was confirmed by the presence of a vaginal plug or sperm in the vagina on the following morning and the day of confirmed copulation was designated as day 0 of gestation.
- The compounds were injected into pregnant rats in two equally divided doses, 2 hr apart on day 9 of gestation. Immediately following the second dose, 1-2  $\mu\text{Ci}$  of the mineral isotope was administered. The rats were then placed into metabolic cages and their urines were collected and assayed for the mineral isotope content.
- On day 20 of gestation, females were sacrificed for fetuses examination (death, resorption, external congenital malformations).

#### **Results and discussion**

##### *Effects on dams*

Marked maternal body weight loss during the advanced periods of pregnancy was observed with salicylic acid. Salicylic acid also induced loss of appetite, complex relaxation, weakness, drowsiness, muscular limpness, inactivity, accelerated respiration rate, and occasionally elevated water intake and urinary excretion.

##### *Effects on offspring*

High incidence of both fetal malformations (5.3%) and resorption (46.6%) were observed with salicylic acid (Table 1). It also causes abnormally small fetuses.

TABLE 1. EFFECTS OF DRUGS AND RELATED COMPOUNDS ON THE RAT FETUSES

	Dose (mg/kg)	No. of rats	Dead mothers after treatment	Total no. of implantations	Mean fetus wt (g)	No. of fetuses (alive)	Resorption		Malformed fetuses	
							No.	Per cent of total implantations	No.	Per cent of total living fetuses
Control	380	15	0	172	3.89 ± 0.44	169	3	1.7	0	0
Aspirin	380	10	0	114	3.02 ± 0.70*	93	21	18.4	18	19.7
Salicylic acid	380	17	1	178	3.09 ± 0.93*	95	83	46.6	5	5.3
<i>m</i> -Hydroxybenzoic acid	380	10	0	123	3.96 ± 0.43	120	3	2.4	0	0
<i>p</i> -Hydroxybenzoic acid	380	10	0	106	3.85 ± 0.31	103	3	2.8	0	0
Anthranilic acid	380	10	0	117	4.02 ± 0.47	112	5	4.2	0	0
Thiosalicylic acid	170	10	0	89	3.84 ± 0.24	84	5	5.6	0	0
Salicylamide	380	10	0	116	3.71 ± 0.53	113	3	2.6	0	0
2,3-Dihydroxybenzoic acid	380	10	0	127	4.12 ± 0.52	117	10	7.8	0	0
2,4-Dihydroxybenzoic acid	380	10	0	115	3.76 ± 0.46	111	4	3.4	0	0
2,5-Dihydroxybenzoic acid	380	12	2	107	3.88 ± 0.61	95	12	11.2	0	0
2,3,4-Trihydroxybenzoic acid	380	10	0	106	3.82 ± 0.44	103	3	2.8	0	0
2,3,5-Trihydroxybenzoic acid	380	10	0	104	4.13 ± 0.35	101	3	2.9	0	0
2,4,6-Trihydroxybenzoic acid	380	11	1	113	3.72 ± 0.70	109	4	3.5	0	0
Salicyluric acid	380	10	0	124	3.98 ± 0.56	121	3	2.4	0	0
Na <sub>2</sub> -EDTA	380	10	0	115	3.99 ± 0.33	30	85	73.0	0	0

\* Significantly different from control, P < 0.01.

RICHARD P. KOSHAKI and ARTHUR R. SCHULERT

### 3.2.2.4 Fritz and Giese (1990)

#### *Study reference:*

Fritz H & Giese K (1990) Evaluation of the teratogenic potential of chemicals in the rat. Pharmacology 40 (suppl 1):1-28.

#### *Detailed study summary and results:*

#### *Test type*

Equivalent or similar to OECD Guideline 414.

#### *Test substance*

Sodium salicylate

#### *Test animals*

- Sprague-Dawley rats
- 17-19 females/dose

#### *Administration/exposure*

- Gavage with 30, 90 or 180 mg/kg (nominal concentration)
- Vehicle: water
- Treatment from gestation day 6 to 15; daily

#### *Description of test design:*

- 2 month old females weighing 200 g were housed overnight with males. Copulation was confirmed by the presence of a vaginal plug or sperm in the vaginal smear on the following morning and the day of confirmed copulation was designed as day 0 of gestation.
- Parameters in dams examined on gestation day 21: body weight, food consumption, ovaries and uterine content.
- Parameters in fetuses examined on gestation day 21: external examination (all fetuses per litter), soft tissue examination (1/3 fetuses per litter), skeletal examination (2/3 fetuses per litter).
- Statistics: Chi 2 test or t test

- Historical control population were given. Data were collected over the period of 63 months and based upon 750 litters.

**Results and discussion**

*Effects on dams*

At 180 mg/kg sodium salicylate, some reduction in food consumption was observed.

*Effects on fetuses*

At 180 mg/kg sodium salicylate, a marked increase in the embryo- and fetolethality was observed (Table 1). At this dose, 30% of the fetuses had malformations, mainly cranio(rachi)schisis (22.7% of the fetuses). There was a dose-related delay in growth at 90 and 180 mg/kg sodium salicylate, indicated by a diminished body weight (Table 1) and a retarded skeletal maturation (Table 2).

**Table 1 : Sodium salicylate: embryotoxicity and teratogenicity following maternal oral treatment on days 6 -15 of pregnancy.**

dosage mg/kg	number of females with implantation sites	mean of implantation sites per female	number of females with abortions	embryo and fetolethality, mean % per dam	Number of live fetuses		mean body weight of live fetuses,g
					males	females	
0	19	14.8	0/19	4.6	127	141	5.41 +/-0.42
30	17	15.4	0/17	5.0	121	128	5.42 +/-0.45
90	19	16.1	0/19	4.9	154	136	5.25 +/-0.41**
180	17	15.1	1/17	29.0*	93	88	3.86 +/-0.67**

\* Chi2 test, p<0.01.

\*\* t test, one-tailed, p<0.01.

**Table 2: sodium salicylate: delay of skeletal maturation as stated for the near-term fetuses**

		dosage			
		0	30 mg/kg	90 mg/kg	180 mg/kg
number of skeletons examined		180	165	192	123
ossification still absent in phalangeal nuclei, %	fore-limb	5.6	7.9	26.0a	93.5a
	hind-limb	43.9	41.2	77.6a	100a
5th sternebra stil incompletely ossified, %		6.7	8.5	5.7	78.9a
some centres of thoracic vertebrae dumbbell-shaped, %		1.1	1.2	10.9a	45.5a
some thoracic vertebrae with 2 small ossified centres, %		0	0	4.7a	83.7a

<sup>a</sup> outside 99% confidence limits of control incidences

**3.2.2.5 Fabro et al. (1984)**

*Study reference:*

Fabro S, McLachlan JA, Dames NM (1984). Chemical exposure of embryos during the preimplantation stages of pregnancy: Mortality rate and intrauterine development. Am J Obstet Gynecol 148:929-938.

*Detailed study summary and results:*

*Test type*

No guideline followed.

**Test substance**

Sodium salicylate

**Test animals**

- New Zealand White rabbit (B&H rabbitry, Rockville, maryland)
- 4 females
- Age: young adult
- Weight at study initiation: 3-4 kg

**Administration/exposure**

- Gavage with 100 mg/kg (actual ingested)
- Vehicle: water
- Treatment from gestation day 4 to 7; daily

**Results and discussion**

At gestation day 8, the implantation ratio was not significantly modified.

Preimplantation treatment did not affect the implantation ratio or the average litter size of viable offspring when litters were examined on gestation day 28 (Table 1). There was no evidence of teratogenic effects.

**Table 1: Effect of sodium salicylate on offspring**

Substance	Sodium salicylate	Control
No of dose	4	22
Total corporea lutea	30	175
Total implantations	25	160
Normal foetuses	20	141
Malformed foetuses	0	0
Litter size	5.0±1.5	6.4±0.5
Foetal weight (g)	35.5±2.3	37.4±1.4

**3.2.2.6 FDA (2006b)**

**Study reference:**

FDA. Center for Drug Evaluation and Research. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% 1-menthol Topical patch). NDA number 22-029. December 13, 2006

The description below is taken from Annex I to the CLH Report of methyl salicylate.

**Detailed study summary and results:**

**Test type**

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

**Test substance**

- Methyl salicylate
- Lot No. Y096
- 1001.1%

**Test animals**

- New Zealand White Rabbit // Kbs:NZW
- 20-22 females/group
- Age: 6 months
- Body weight on gestation day 0 ranged from 3.384 kg to 3.998 kg

Group	Test Article	Dosage Level (mg/kg/day)	Dosage Concentration (mg/mL)	Dosage Volume (mL/kg)	Number of Females
1	Methyl Salicylate	0	0	1.0	22
2	Methyl Salicylate	30	30	1.0	20
3	Methyl Salicylate	100	100	1.0	21
4	Methyl Salicylate	300	300	1.0	20

**Administration/exposure**

- 30, 100, 300 mg/kg/day. The high dose was based on a preliminary study for effects on embryo-fetal development (dosage: 29, 83, 250, 500 and 750 mg/kg). In this study, death was observed in the dams in the 500 and 750 mg/kg groups and an increase in the pre-implant loss index was observed in the 250 and 500 mg/kg groups. The high dose for the main study was thus set at 300 mg/kg/day, which it was expected to cause no death of dams and to develop some toxicological signs. The middle and low doses were set at 100 and 30 mg/kg, respectively, in a common ratio of about 3.
- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The stability of the test article during administration period was confirmed according to the results of analyses of the test article conducted periodically. The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepared for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within 100 ± 5%)
- The test article mixture was administered to the dorsal subcutis using a needle (22G) and syringe once daily for 13 days from day 6 to day 18 of gestation. The actual dosage volume was individually calculated from the body weight on day 6 of gestation.

**Description of test design:**

- Mating was conducted between males and females at the ages of 6-7 and 5-6 months, respectively. Females with full receptivity based on vulva signs were housed together with males in the male cages. The successful copulation was confirmed by the presence of sperm in the vaginal smear. The day of successful copulation was designated as day 0 of gestation.
- The test article mixture was administered to the dorsal subcutis using a needle (22G) and syringe once daily for 13 days from day 6 to day 18 of gestation.

- Parameters in dams: clinical observation (twice daily during the administration period and once daily during other period), body weight (measured on days 0, 3, 6-19, 23, 26 and 29 of gestation), body weight gain and food consumption (measured on days 0, 3, 6-19, 23, 26 and 29 of gestation), necropsy (organs and tissues examined macroscopically; skin of the treated site, ovaries and uterus and organs and tissues with lesions were fixed in 10% neutral buffered formalin solution),
- Parameters in embryos and foetuses: number of corpora lutea, implants, early and late resorptions, dead and live foetuses were recorded, placenta was observed macroscopically, live foetuses were weighted individually and examined for external anomalies (including the oral cavity). All the live foetuses were examined macroscopically and sexed; they were sectioned into head, chest and abdomen, the brain, kidney and heart were examined for visceral anomalies; other organs were individually fixed in 10% neutral buffered formalin solution and preserved. All the carcasses of the foetuses were examined for skeletal anomalies, variations and progress of ossification (stained with Alizarin red S).
- Determination of plasma salicylic acid concentration: blood was collected 1 time at 4 hours after administration on days 6 and 18 of gestation. The concentration of salicylic acid was measured by HPLC.

**Results and discussion**

- As regards the body weight, body weight gain, food consumption, numbers of corpora lutea, implants, live foetuses, vertebral bodies and arches, and body weight of the live foetuses, the mean and standard deviations were calculated for every group, and the homogeneity of variance was tested by Bartlett’s method. Comparison of the treated groups with the control group was made by Dunnett’s method when the variance was found to be homogeneous or by Steel’s method when the variance was not homogeneous. The indices of pre-implant loss, early and late resorption dead foetuses, total dead foetuses, placental (by type) anomalies, external (by type) anomalies, visceral (by type) variation and progress of ossification were analyzed by Wilcoxon’s rank sum test. Levels of P <0.01 and P<0.05 were considered to be significant in all cases. The values for foetuses were recorded with each litter treated as unit.

*Effects on dams:*

- One dam in the 300 mg/kg group had an abortion on day 18 of gestation, and extensive vaginal haemorrhaging, blanching and pale eyes were then observed in this dam. A decrease in body weight and poor food consumption were also observed in this dam beginning on day 14 of gestation. In addition, crust of the treated site or loss of hair were observed in 1 dam in the 100 mg/kg group from day 14 to day 29 of gestation.

**Summary of Clinical Signs**

Rabbit №	Dose (mg/kg/day)	Day of Occurrence	Observed Clinical Sign(s)	Outcome	Necropsy Results
318	100	Gestation days 14 - 25	Crust at treatment site		
		Gestation Day 26-29	Loss of hair		
415	300	Gestation Day 18	Blanching Vaginal hemorrhage Abortion		<b>Uterus:</b> Retention of bloody material; <b>Vagina:</b> Retention of bloody material <b>Subcutis (treated and untreated site):</b> Retention of oily fluid All fetuses in the uterus had died (late resorption)

- There was no significant difference on body weight between methyl salicylate groups and the control group. A depressive trend in body weight gain (not statistically significant) was observed throughout the administration period in the 300 mg/kg group as compared with the control group.

Table 3 Body weight gains in Fo dams

Group and dose		Control			30 mg/kg			100 mg/kg			300 mg/kg		
Days of gestation		Body weight gain (g)											
7		-4±	36	(20)	2±	31	(20)	-5±	30	(19)	-23±	55	(18)
8		14±	46	(20)	14±	45	(20)	4±	44	(19)	-9±	80	(18)
9		22±	53	(20)	29±	40	(20)	16±	43	(19)	-9±	72	(18)
10		48±	63	(20)	48±	48	(20)	44±	47	(19)	16±	81	(18)
11		55±	49	(20)	79±	54	(20)	60±	56	(19)	19±	78	(18)
12		79±	69	(20)	90±	52	(20)	82±	55	(19)	40±	81	(18)
13		105±	71	(20)	118±	51	(20)	115±	69	(19)	63±	87	(18)
14		137±	92	(20)	140±	59	(20)	148±	66	(19)	110±	103	(18)
15		157±	94	(20)	160±	82	(20)	167±	77	(19)	130±	118	(18)
16		169±	109	(20)	198±	69	(20)	196±	74	(19)	138±	178	(18)
17		199±	100	(20)	203±	78	(20)	218±	74	(19)	128±	214	(18)
18		209±	95	(20)	228±	89	(20)	235±	87	(19)	185±	110	(17)
19		221±	93	(20)	228±	93	(20)	228±	101	(19)	189±	140	(17)
23		244±	135	(20)	247±	116	(20)	260±	133	(19)	233±	146	(17)
26		258±	161	(20)	244±	146	(20)	268±	177	(19)	236±	180	(17)
29		295±	193	(20)	254±	181	(20)	284±	228	(19)	248±	245	(17)

Not significantly different from control.  
 Values are mean±S.D. and the values in parentheses represent the number of dams.  
 One animal (No. 415) of the 300 mg/kg group had an abortion on day 18 of gestation.

- A significant increase in food consumption as compared with the control group was observed on day 1 of gestation in the 30 mg/kg group and on days 1-7, 9-10, 14 and 16 of gestation in the 100 mg/kg group. This effect was not dose-related.
- In necropsy of the dams on day 29 of gestation, retention of oily fluid in the subcutis of the treated site or the non-treated site (breast, axillary region or abdomen) was observed in all the dams in the control and methyl salicylate groups. Loss of hair in the treated site was observed in 1 dam in the 100 mg/kg group. In addition, light gray macule in the lung was observed in 1 dam in the 300 mg/kg group, but this change was considered to be incidental change not related to administration of methyl salicylate since it showed a low incidence. In necropsy of the dam that had an abortion, retention of oily fluid in the subcutis of the treated site and the non-treated site (axillary region) was observed. In addition, retention of bloody material in the vagina and uterus was observed, and all the foetuses in the uterus had died (late resorption).

*Effects on embryo-fetal development:*

- There was a significant difference in sex ratio, with a larger number of male foetuses in the 300 mg/kg group as compared with the control group (↑ 44.4%). In addition, a significant decrease in the pre-implant loss index (66.7%) as compared with the control group was observed in the 30 mg/kg group. This effect was not dose-related. There was no significant effect on the numbers of corpora lutea, implants or live foetuses, total dead foetuses, early resorption, late resorption and dead foetus indices or body weights of live foetuses. No abnormality was observed in the external examination or macroscopic observation of the placenta in the control or methyl salicylate groups.

Table 7 Observation on cesarean section of Fo dams

Group and dose	Control	30 mg/kg	100 mg/kg	300 mg/kg
No. of dams	20	20	19	17
No. of corpora lutea a)	181 ( 9.05 ± 2.09)	186 ( 9.30 ± 1.89)	158 ( 8.32 ± 2.21)	151 ( 8.88 ± 1.58)
No. of implants a)	151 ( 7.55 ± 2.52)	176 ( 8.80 ± 1.77)	126 ( 6.63 ± 2.85)	133 ( 7.82 ± 2.19)
No. of pre-implant loss b)	30 (16.57)	10 ( 5.38)*	32 (20.25)	18 (11.92)
No. of total dead fetuses c)	7 ( 4.64)	18 (10.23)	7 ( 5.56)	11 ( 8.27)
Early resorptions	4 ( 2.65)	9 ( 5.11)	5 ( 3.97)	6 ( 4.51)
Late resorptions	3 ( 1.99)	9 ( 5.11)	2 ( 1.59)	5 ( 3.76)
Dead fetuses	0	0	0	0
No. of live fetuses a)	144 ( 7.20 ± 2.61)	158 ( 7.90 ± 1.74)	119 ( 6.26 ± 2.88)	122 ( 7.18 ± 2.24)
Sex ratio of live fetuses d)	0.95 ( 70/ 74)	1.05 ( 81/ 77)	1.16 ( 64/ 55)	1.71 ( 77/ 45)*
Body weight of live fetuses (g) e)				
Male	40.85 ± 7.40	37.62 ± 5.33	40.73 ± 7.78	39.69 ± 6.52
Female	39.74 ± 5.90	37.89 ± 6.93	39.85 ± 8.33	38.23 ± 7.42
No. of live fetuses with external anomalies	0	0	0	0
No. of live fetuses with placental anomalies	0	0	0	0

\*: P<0.05 (significantly different from control).

a) Values in parentheses represent mean±S.D.

b) Values in parentheses represent percentages to the number of corpora lutea.

c) Values in parentheses represent percentages to the number of implants.

d) Values in parentheses represent number of male/female fetuses.

e) Values are mean±S.D.

- Hypoplastic lung was observed in 1 fetus in the 300 mg/kg group and thymic remnant in the neck was observed in 1 fetus in the control group. There was no significant difference, however, between the incidences in the control and 300 mg/kg groups. No visceral anomalies were observed in the 30 and 100 mg/kg groups.
- Fetuses with skeletal anomalies were observed in 3 (2.08%), 8 (5.06%), 2 (1.68%) and 3 (2.46%) foetuses in the control, 30, 100 and 300 mg/kg groups respectively, but there was no significant difference between the incidences in the control and methyl salicylate groups. Considered by type of anomaly, nodulated rib was observed in 1 fetus each in the control, 100 and 300 mg/kg groups and fusion of the sternebra was observed in the 2, 8, 1 and 2 fetuses in the control, 30, 100 and 300 mg/kg groups, respectively. There was no significant difference, however, between the control and methyl salicylate groups in terms of incidences of these anomalies by type.
- Fetuses with skeletal variation were observed in 128 (88.89%), 115 (72.78%), 100 (84.03%) and 90 (73.77%) foetuses in the control, 30, 100 and 300 mg/kg groups, respectively, but there was no significant difference between these incidences in the control and methyl salicylate groups. Considered by type of variation, cervical rib was observed in 1 fetus each in the 100 and 300 mg/kg groups; a short supernumerary rib was observed in 5, 11, 4 and 4 fetuses in the control, 30, 100 and 300 mg/kg groups, respectively; a full supernumerary rib was observed in 124, 111, 99 and 86 fetuses in the control, 30, 100 and 300 mg/kg groups, respectively; asymmetry of the sternebra was observed in 1 fetus in the 300 mg/kg group; splitting of the sternebra was observed in 1, 3, 3 and 2 fetuses in the control, 30, 100 and 300 mg/kg group; and incomplete ossification of lumbar or caudal centrum in 1, 3 and 1 fetuses in the control, 30 and 300 mg/kg groups, respectively. There was no significant difference between the control and methyl salicylate groups in the incidences of these variations by type. No significant difference in the progress of ossification of the vertebra, sternebra and metacarpus or phalanges was observed between the control and the methyl salicylate groups.

Table 9 Skeletal examinations in F<sub>1</sub> fetuses

Group and dose	Control	30 mg/kg	100 mg/kg	300 mg/kg
No. of fetuses examined	144	158	119	122
No. of fetuses with skeletal anomalies	3 ( 2.08)	8 ( 5.06)	2 ( 1.68)	3 ( 2.46)
Nodulated rib	1 ( 0.69)	0	1 ( 0.84)	1 ( 0.82)
Fusion of the sternebra	2 ( 1.39)	8 ( 5.06)	1 ( 0.84)	2 ( 1.64)
No. of fetuses with skeletal variations	128 (88.89)	115 (72.78)	100 (84.03)	90 (73.77)
Cervical rib	0	0	1 ( 0.84)	1 ( 0.82)
Short supernumerary rib	5 ( 3.47)	11 ( 6.96)	4 ( 3.36)	4 ( 3.28)
Full supernumerary rib	124 (86.11)	111 (70.25)	99 (83.19)	86 (70.49)
Asymmetry of the sternebra	0	0	0	1 ( 0.82)
Splitting of the sternebra	1 ( 0.69)	3 ( 1.90)	3 ( 2.52)	2 ( 1.64)
Accessory sternebra	0	1 ( 0.63)	0	0
Incomplete ossification of lumbar centrum	0	0	0	1 ( 0.82)
Incomplete ossification of caudal centrum	1 ( 0.69)	3 ( 1.90)	0	0

Not significantly different from control.

Values in parentheses represent percentages to the number of fetuses examined.

- The plasma salicylic acid concentrations at 4 hours after administration in the 30, 100 and 300 mg/kg groups were 24.3, 62.5 and 142 µg/ml on day 6 of gestation (the first administration) and 16.5, 47.8 and 98.4 µg/ml on day 18 of gestation (the final administration), respectively.

### 3.2.2.7 FDA (2006c)

#### *Study reference:*

FDA. Center for Drug Evaluation and Research. 2006. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% l-menthol Topical patch). NDA number 22-029.

The description below is taken from Annex I to the CLH Report of methyl salicylate.

#### *Detailed study summary and results:*

##### *Test type*

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

##### *Test substance*

- Methyl salicylate
- Lot No. Y096
- 1001.1%

##### *Test animals*

- Crj:CD(SD)IGS rats
- 20 females/groups
- The body weights on day 0 of gestation were 247.5-299.2 g

##### *Administration/exposure*

- 0, 50, 100, 200 mg/kg/day. The high dose was based on a preliminary study for effects on embryo-fetal development (dosage: 75, 150, 300 and 400 mg/kg). In this study, mortality was observed in the dams in the 400 mg/kg group and a depression of body weight gain in dams, lethal on embryos, teratogenicity and suppression of fetal growth in the groups receiving 300 mg/kg or above were observed. The high dose for the main study was thus set at 200 mg/kg/day, which was expected to cause no mortality of dams and embryos and to develop some toxicological signs. The middle and low doses were set at 100 and 50 mg/kg, respectively, in a common ratio of about 2.

- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The stability of the test article during administration period was confirmed according to the results of analyses of the test article conducted periodically. The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepared for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within  $100 \pm 5\%$ ).
- The test article mixture was administered to the dorsal subcutis using a needle (26G) and syringe once daily for 12 days from day 6 to day 17 of gestation. The actual dosage volume was individually calculated from the body weight on day 6 of gestation.

### ***Description of test design:***

- Mating was started at 12 weeks of age. Nulliparous females were housed overnight with males in a 1:1 ratio. The successful copulation was confirmed by the presence of sperm in the vaginal smear. The day of successful copulation was designated as day 0 of gestation.
- The test article mixture was administered by subcutaneous injection once daily for 12 days from day 6 to day 17 of gestation.
- Parameters in dams: clinical observation (twice daily during the administration period and once daily during other period), body weight (measured on days 0, 3 and 6-20 of gestation), body weight gain and food consumption (measured on days 1, 3 and 6-20 of gestation), necropsy (organs and tissues examined macroscopically; skin of the treated site, ovaries and uterus were fixed in 10% neutral buffered formalin solution).
- Parameters in embryos and foetuses: number of corpora lutea, implants, early and late resorptions, dead and live foetuses were recorded, placenta was observed macroscopically, live foetuses were weighted individually, sexed and examined for external anomalies (including the oral cavity). Approximately half of the foetuses from each litter were identified individually by dorsal number with an oily felt pen and fixed in Bouin's solution. The other half of the foetuses were identified individually by tattooing the four limbs after removal of the organs from the chest and abdomen and fixed in 70% ethanol. The live foetuses with external anomalies were fixed in 10% neutral buffered formalin.
- Visceral anomalies were observed in the foetuses in the control and 200 mg/kg groups that were fixed in Bouin's solution by the razor blade section method for the head and abdomen and by the microdissection method for the chest.
- The foetuses that were fixed in 70% ethanol were stained with Alizarin red S and these foetuses in all groups, including the control were examined for skeletal anomalies, variations and progress of ossification with a stereoscopic microscope.

### ***Results and discussion***

- As regards body weight, body weight gain, food consumption, numbers of corpora lutea, implants, live foetuses, vertebral bodies and arches, and body weight of the live foetuses, the mean and standard deviations were calculated for every group, and the homogeneity of variance was tested by Bartlett's method. Comparison of the treated groups with the control group was made by Dunnett's method when the variance was found to be homogeneous or by Steel's method when the variance was not homogeneous. The sex ratios of live foetuses were analyzed by the  $\chi^2$  test. The indices of pre-implant loss, early and late resorption, dead foetuses, total dead foetuses, placental (by type) anomalies, external (by type) anomalies, visceral (by type) anomalies, skeletal (by type) anomalies and skeletal (by type) variations and progress of ossification were analyzed by Wilcoxon's rank sum

test. Levels of  $P < 0.01$  and  $P < 0.05$  were considered to be significant in all cases. The values for foetuses were recorded with each litter treated as unit.

*Effects on dams:*

- No mortality occurred in the control or methyl salicylate groups. There were no abnormal signs in the control or methyl salicylate groups.
- A significant lower mean body weight as compared with the control group was observed on days 7 (3.5%), 8 (4.2%), 9 (3.4%), 10 (3.9%), 12 (3.4%) and 13 (3.6%) of gestation in the 200 mg/kg group.
- A significant decrease of body weight gain as compared with the control group was observed on day 7 of gestation in the 100 mg/kg group and during the whole treatment in the 200 mg/kg group. This decrease was  $\geq 10\%$  throughout gestation.

Day of Treatment	Vehicle Control	Mean Body Weight Gain (g) $\pm$ SD in F <sub>0</sub>	
		200 mg/kg/day	% Change of Control
7	3.5 $\pm$ 2.7	-7.5 $\pm$ 4.6**	314.3%%
8	8.7 $\pm$ 5.8	-4.8 $\pm$ 6.1**	155.2%
9	12.7 $\pm$ 4.0	1.4 $\pm$ 5.3**	89%
10	19.2 $\pm$ 4.8	6.1 $\pm$ 8.3**	68%
11	23.0 $\pm$ 5.5	12.0 $\pm$ 7.6**	47.8%
12	30.3 $\pm$ 6.1	18.6 $\pm$ 5.7**	38.6%
13	35.2 $\pm$ 5.5	22.4 $\pm$ 8.7**	36.4%
14	40.7 $\pm$ 5.7	30.0 $\pm$ 9.1**	24.1%
15	48.4 $\pm$ 5.9	37.9 $\pm$ 7.1**	21.2%
16	58.2 $\pm$ 7.6	48.2 $\pm$ 8.2**	17.2%
17	71.1 $\pm$ 7.9	62.5 $\pm$ 9.2**	12.1%
18	88.0 $\pm$ 10.4	76.6 $\pm$ 10.2**	13%
19	103.7 $\pm$ 11.5	93.1 $\pm$ 11.4*	10.2%
20	120.5 $\pm$ 12.2	106.4 $\pm$ 13.1**	11.7%

\* Significantly different from vehicle control ( $p < 0.05$ )

\*\* Significantly different from vehicle control ( $p < 0.01$ )

- A significant decrease in food consumption as compared with the control group was observed on day 7 of gestation (-11.6%) in the 100 mg/kg group and on days 6, 7 and 8 of gestation in the 200 mg/kg group (5%, 25.7% and 18.2% respectively).
- In the necropsy of the dams on day 20 of gestation, retention of oily fluid in the subcutis of the treated site was observed in all the dams in the control and methyl salicylate groups.

*Effects on embryo-fetal development:*

- A significant lower body weight of live foetuses as compared with the control group was observed in the 200 mg/kg group (mean body weight: -22%).
- There were no significant differences between the control and methyl salicylate groups in the numbers of corpora lutea, implants or live foetuses, pre-implant loss, total dead foetuses, early resorption, late resorption, or dead fetus indices or sex ratio.
- In the external examination, foetuses with external anomalies were observed in 1 (0.36%) and 9 (3.21%) foetuses in the control and 200 mg/kg groups, respectively, but there was no significant difference between the incidences in the control and 200 mg/kg groups. Considered by type of anomaly, craniorachischisis was observed in 8 fetuses from 3 litter in the 200 mg/kg group and gastroschisis was also observed in 1 fetus among them; oedema was observed in 1 fetus from another litter in the 200 mg/kg group; and vestigial tail was observed in 1 fetus from 1 litter in the control group. There was no significant difference, however, between the control and 200 mg/kg groups in terms of incidences of these anomalies by type. No abnormality was observed in the macroscopic observation of the placenta in the control or methyl salicylate groups.

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Table 6 Observation on cesarean section of Fo dams

Group and dose	Vehicle control	50 mg/kg	100 mg/kg	200 mg/kg
No. of dams	20	20	20	20
No. of corpora lutea a)	306 (15.30 ± 1.89)	319 (15.95 ± 0.83)	304 (15.20 ± 1.36)	305 (15.25 ± 1.33)
No. of implants a)	296 (14.80 ± 2.33)	304 (15.20 ± 2.55)	289 (14.45 ± 2.50)	300 (15.00 ± 1.30)
No. of pre-implant loss b)	10 ( 3.27)	15 ( 4.70)	15 ( 4.93)	5 ( 1.64)
No. of total dead fetuses c)	21 ( 7.09)	17 ( 5.59)	10 ( 3.46)	20 ( 6.67)
Early resorptions	21 ( 7.09)	17 ( 5.59)	10 ( 3.46)	20 ( 6.67)
Late resorptions	0	0	0	0
Dead fetuses	0	0	0	0
No. of live fetuses a)	275 (13.75 ± 2.22)	287 (14.35 ± 2.58)	279 (13.95 ± 2.54)	280 (14.00 ± 1.81)
Sex ratio of live fetuses d)	1.15 (147/128)	0.98 (142/145)	1.01 (140/139)	1.31 (159/121)
Body weight of live fetuses (g) e)				
Male	3.64 ± 0.36	3.65 ± 0.32	3.57 ± 0.30	2.84 ± 0.32**
Female	3.45 ± 0.35	3.45 ± 0.21	3.34 ± 0.27	2.68 ± 0.21**
No. of live fetuses with external anomalies f)	1 ( 0.36)	0	0	9 ( 3.21)
Craniorachischisis	0	0	0	8 ( 2.86)
Edema	0	0	0	1 ( 0.36)
Gastroschisis	0	0	0	1 ( 0.36)
Vestigial tail	1 ( 0.36)	0	0	0
No. of live fetuses with placental anomalies	0	0	0	0

\*\* : P<0.01 (significantly different from vehicle control).

a) Values in parentheses represent mean±S.D.

b) Values in parentheses represent percentages to the number of corpora lutea.

c) Values in parentheses represent percentages to the number of implants.

d) Values in parentheses represent number of male/female fetuses.

e) Values are mean±S.D.

f) Values in parentheses represent percentages to the number of live fetuses.

- Fetuses with visceral anomalies were observed in 5 (3.52%) and 11 (7.75%) foetuses in the control and 200 mg/kg groups, respectively, but there was no significant difference between the incidences in the control and 200 mg/kg groups. Considered by type of anomaly, ventricular septal defect was observed in 1 fetus in the 200 mg/kg group; dilatation of the ureter (unilateral) was observed in 3 and 2 fetuses in the control and 200 mg/kg groups, respectively; and thymic remnant in the neck was observed in 2 and 8 fetuses in the control and 200 mg/kg groups, respectively. There was no significant difference, however, between the control and 200 mg/kg groups in terms of incidence of these anomalies by type.
- Skeletal anomalies were observed in 3 (2.24%) and 2 (1.55%) foetuses in the 100 and 200 mg/kg groups, respectively, but there was no significant difference between the incidences in the control and methyl salicylate groups. Considered by type of anomaly, wavy ribs were observed in 3 and 1 fetuses in the 100 and 200 mg/kg groups, and fusion of the ribs was observed in 1 fetus in the 200 mg/kg group, respectively. There was no significant difference, however, between the control and methyl salicylate groups in terms of incidences of these anomalies by type.
- Skeletal variations were observed in 14 (10.61%), 27 (19.71%), 20 (14.93%) and 97 (75.19%) fetuses in the control, 50, 100 and 200 mg/kg groups, respectively, and a significant increase in the incidence was observed in the 200 mg/kg group as compared with the control group. Considered by type of variation, cervical rib was observed in 2 and 1 fetuses in the 100 and 200 mg/kg groups; a short supernumerary rib was observed in 12 (9.09%), 23 (16.79%), 18 (13.43%) and 43 (33.33%) fetuses in the control, 50, 100 and 200 mg/kg groups, respectively; a full supernumerary rib was observed on 59 (45.74%) fetuses in the 200 mg/kg group; asymmetry of the sternebra was observed in 1 and 2 fetuses in the 50 and 200 mg/kg groups, respectively; splitting of the sternebra was observed in 1 and 3 fetuses in the 100 and 200 mg/kg groups, respectively; splitting of the thoracic vertebral body was observed in 15 (11.63%) fetuses in the 200 mg/kg group; 7 lumbar vertebra was observed in 42 (32.56%) fetuses in the 200 mg/kg group. Incomplete ossification of the thoracic centrum was observed in the 200 mg/kg group as compared with the control group. In the progress of ossification of the vertebrae, sternebra, metacarpus, metatarsus and phalanges, a significant decrease in the numbers of cervical vertebral bodies, thoracic vertebral bodies and sacrocaudal vertebral arches and bodies, and ossification indices of the 6<sup>th</sup> sternebra, metacarpus, metatarsus, proximal phalanges of the forelimb and distal phalanges of the hindlimb and a significant increase in

the numbers of the lumbar vertebral arches and bodies were observed in the 200 mg/kg group as compared with the control group.

SKELETAL EXAMINATIONS		
Parameter	Dose (mg/kg/day)	
	0	200
No litters examined skeletally	132	129
	<b>SKELETAL VARIATIONS</b> Number of Subjects (percentage of the fetuses examined)	
No of offsprings with skeletal variations (%)	14 (10.61)	97 (75.19)**
Cervical rib	0	1 (0.78)
Short supernumerary rib	12 (9.09)	43 (33.33)**
Full supernumerary rib	0	59 (45.74)**
Asymmetry of the sternebra	0	2 (1.55)
Splitting of the sternebra	0	3 (2.33)
Splitting of the thoracic vertebral body	3 (2.270)	42 (32.56)**
Splitting of the lumbar vertebral body	0	15 (11.63)**
7 lumbar vertebrae	0	42 (32.56)**
In complete ossification of thoracic centrum	0	10 (7.75)*
In complete ossification of lumbar centrum	0	1 (0.78)
	<b>PROGRESS OF OSSIFICATION</b> Mean ( $\pm$ S.D.) or Number of ossification (%) <sup>A</sup>	
<b>VERTBRAE</b>		
Cervical (body)	0.89 (0.89)	0.08 (0.15)**
Thoracic (body)	12.98 (0.070)	12.69 (0.49)**
Lumbar		
- Arch (R)	6.0 (0.0.)	6.33 (0.44)**
- Arch (L)	6.0 (0.0)	6.33 (0.44)**
- Body	6.0 (0.0)	6.33 (0.44)**
Sacrocaudal		
- Arch (R)	5.94 (0.36)	5.61 (0.47)*
- Arch (L)	5.95 (0.36)	5.60 (0.49)*
- Body	7.90 (0.72)	6.91 (0.76)**
<b>STERNEBRAE</b>		
- 6 <sup>th</sup>	130 (98.48) <sup>A</sup>	92 (72.32) <sup>A</sup> **
<b>METACARPUS</b>		
- Right	513 (77.73) <sup>A</sup>	406 (62.95) <sup>A</sup> **
- Left	513 (77.73) <sup>A</sup>	394 (61.56) <sup>A</sup> **
<b>PHALANGES OF HINDLIMBS</b>		
- Distal	640 (98.46) <sup>A</sup>	614 (95.19) <sup>A</sup> **
<b>METATARSUS</b>		
- Right	540 (82.44) <sup>A</sup>	509 (79.53) <sup>A</sup> **
- Left	546 (82.73)	515 (79.84) <sup>A</sup> *

\*: p < 0.05, significantly different from control  
 \*\*: p < 0.01, significantly different from control  
 A: represent the number of ossification (ossification percentage)

### 3.2.2.8 FDA (2006d)

#### Study reference:

FDA. Center for Drug Evaluation and Research. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% l-menthol Topical patch). NDA number 22-029. December 13, 2006

The description below is taken from Annex I to the CLH Report of methyl salicylate.

#### Detailed study summary and results:

#### Test type

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

#### Test substance

- Methyl salicylate
- Lot No. Y096

- 1001.1%

### *Test animals*

- Crj:CD(SD)IGS rats
- 20 females/groups
- The body weights on day 0 of gestation were 240.8-305.1 g

### *Administration/exposure*

- 0, 20, 60, 200 mg/kg/day. The high dose was based on a preliminary study for effects on pre- and postnatal development, including maternal function (dosage: 32, 80, 200, 300 and 500 mg/kg). In this study, mortality was observed in almost all the dams in the 500 mg/kg group. Almost none of the dams in the 300 mg/kg group delivered due to a lethal on the embryos. Furthermore, a decrease in the birth index or a decrease in the body weights of live newborns were observed in the 80 or 200 mg/kg groups, but the degree of these effects was slight. The high dose for the main study was thus set at 200 mg/kg/day, which it was expected to cause no mortality of dams and embryos and to develop some toxicological signs. The middle and low doses were set at 60 and 20 mg/kg, respectively, in a common ratio of about 3.
- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The stability of the test article during administration period was confirmed according to the results of analyses of the test article conducted periodically. The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepared for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within  $100 \pm 5\%$ )
- The test article mixture was administered to the dorsal subcutis using a needle (26G) and syringe once daily from day 6 of gestation to day 21 of lactation (the delivery day was designated as day 0 of lactation). The actual dosage volume was individually calculated from the body weight on day 6 of gestation during the gestation period and on day 0 of lactation during the lactation period.

### *Description of test design:*

- Mating was started at 12 weeks of age. Nulliparous females were housed overnight with males in a 1:1 ratio. The successful copulation was confirmed by the presence of sperm in the vaginal smear. The day of successful copulation was designated as day 0 of gestation.
- The test article mixture was administered by subcutaneous injection once daily from day 6 of gestation to day 21 of lactation.
- Parameters in dams: clinical observation (twice daily during the administration period and once daily during other period), delivery and nursing conditions, including signs of delivery in the late stage of gestation, gestational days and gestation index, body weight (measured on days 0, 3, 6, 9, 12, 15, 18 and 20 of gestation and days 0, 4, 7, 10, 14, 17 and 21 of lactation), body weight gain and food consumption (measured on days 1, 3, 6, 9, 12, 15, 18 and 20 of gestation and on days 1, 4, 7, 10, 14, 17 and 21 of lactation), necropsy (organs and tissues examined macroscopically; number of implantation traces in the uterus was counted; organs and tissues with lesions, the skin of the treated site, ovaries and uterus were fixed in 10% neutral buffered formalin solution).
- Parameters in offspring:
  - state of delivery, number of litter, stillborns and live newborns were counted after delivery, birth index and stillbirth index were calculated. The live newborns were weighed, sexed and

examined for external anomalies. The stillborns were examined with a floating test of the extracted lungs to determine whether they had breathed or not.

- The litter size was standardized by random removal on postnatal day 4, such that the number of live newborns of each litter was adjusted to 8 with 4 of each sex.
- After birth and until the time of weaning (postnatal day 22), the live newborns were individually weighed on day 0, 4, 7, 14 and 21 after birth and observed for clinical signs and mortality. The viability index and the weaning index were calculated. The live newborns that died during the lactation period were fixed in pure ethanol. After weaning (postnatal day 22) and until mating, the offspring were observed for clinical signs and mortality at least once daily and their body weight and food consumption were measured once weekly. After mating, successfully copulating females were observed daily for clinical signs and their body weights were measured on days 0, 4, 7, 10 and 13 of gestation. The males and unsuccessfully copulating females were observed daily for clinical signs until necropsy.
- On the day of weaning, 3 offspring of each sex from litter containing 4 males and 4 females were necropsied, after which the organs of 1 offspring of each sex among these were weighted and moreover the other 2 offspring of each sex were submitted for skeletal examination. The remaining 1 offspring of each sex was subjected to tests of motor coordination, learning ability and emotional behaviour and to a reproductive performance test.
- All the offspring were examined for pinna detachment on postnatal day 4, piliation on postnatal day 8, incisor eruption on postnatal day 10, gait and eyelid separation on postnatal day 15, descensus testis on postnatal day 21, cleavage of balanopreputial gland on postnatal day 42 and vaginal opening on postnatal day 42. The offspring testing negative were subjected to the tests on a daily basis until they tested positive.
- All the offspring were examined for righting reflex and ipsilateral flexor reflex on postnatal day 5, visual reflex on postnatal day 16 and preyer's reflex using audiometer on postnatal day 28. The offspring testing negative were subjected to the tests on a daily basis until they tested positive.
- On postnatal day 22, 1 offspring of each sex from a dam was sacrificed by exsanguination from the lateral iliac artery under ether anesthesia and its organs and tissues were macroscopically observed. The following organs were then isolated and weighed: heart, lungs, liver, kidneys, adrenals, brain, spleen, thymus and testes or ovaries. The organs and tissues with lesions were fixed in 10% neutral buffered formalin solution with the same organs and tissues from the control group and preserved.
- On postnatal day 22, offspring of each sex from a dam were sacrificed by exsanguination under ether anesthesia and their organs and tissues were observed macroscopically. The organs and tissues with lesions were fixed in 10% neutral buffered formalin solution with the same organs and tissues from the animals from the control group and preserved. After all the carcasses were fixed in 70% ethanol and stained with alizarin red S, these in the control, 60 and 200 mg/kg groups were examined for skeletal anomalies and variations using a stereoscopic microscope.
- At the time the offspring reached 5 weeks of age, their motor coordination was examined with a rotarod by counting the number of falls during 3 minutes from the rod, which rotated 5 times a minute.
- At the time the offspring reached 6 weeks of age, their learning ability was examined with a water maze test, which was conducted for 4 consecutive days.
- At the time the offspring reached 8 weeks of age, their emotionality was examined by Hall's method with an open field and behaviour analyse system.
- On 12-13 weeks of age, the males were paired with the females from the same group on a 1:1 basis to avoid mating within litter. The pairs were observed for successful copulation for

2 weeks. Successful copulation was confirmed by the presence of a vaginal plug or of sperm in a vaginal smear and the day of successful copulation was designed as day 0 of gestation. The duration of mating required for successful copulation, copulation index and male or female fertility indices were calculated.

- The males achieving successful copulation were sacrificed and their organs and tissues were examined macroscopically. The testes and epidymis and the organs and tissues with lesions were fixed in 10% neutral buffered formalin solution. The females achieving successful copulation were sacrificed and their organs and tissues were examined macroscopically after removal of the ovaries and uterus. The organs and tissues with lesions were fixed in 10% neutral buffered formalin solution. For uncopulating females, their organs and tissues were examined macroscopically after the mating period and ovaries and uterus were fixed in 10% neutral buffered formalin solution. Dead animals after weaning were necropsied immediately after body weight measurement and their organs and tissues with lesions were fixed in 10% neutral buffered formalin solution and preserved.
- After removal of the ovaries and uterus, the numbers of corpora lutea, implantation, dead embryos and live embryos were counted and the pre-implantation loss index and dead embryo index were calculated. The ovaries and uterus were fixed in 10% neutral buffered formalin solution.

### ***Results and discussion***

- As regards body weight, body weight gain, food consumption, gestational days, organ weights, numbers of implantation, litter, live newborns, corpora lutea and live embryos and motor coordination, learning ability and emotional behaviour test values and duration (in days) of mating required for successful copulation, the mean and standard deviations were calculated for every group, and the homogeneity of variance was tested by Bartlett's method. Comparison of the treated groups with the control group was made by Dunnett's method when the variance was found to be homogeneous or by Steel's method when the variance was not homogeneous. The gestation, copulation and male or female fertility indices and sex ratios of live foetuses were analysed by the  $\chi^2$  test. The birth, stillbirth, viability, external (by type) anomalies, skeletal (by type) anomaly, skeletal (by type) variations, pre-implantation loss and dead embryo indices and the development of differentiation and function test values were analyzed by Wilcoxon's rank sum test. Levels of  $P < 0.01$  and  $P < 0.05$  were considered to be significant in all cases. The values for offspring obtained before weaning were recorded with each litter treated as unit.

### ***Effects on dams***

- Two dams in the 200 mg/kg group died on day 23 of gestation. Vaginal haemorrhaging was observed in one of these dead dams and the other dam had delivered 4 live newborns and 11 stillborn upon the discovery of their death. No abnormality related to clinical signs occurred in the control or methyl salicylate groups. As regards delivery and nursing conditions, there were no changes in the control or methyl salicylate groups. A significant prolongation of gestational days was observed in the 60 mg/kg group as compared with the control group. No significant difference in the numbers of implantation or the litter and gestation indices was observed in the methyl salicylate groups as compared with the control group.
- During the gestation period, a significantly lower mean body weight as compared with the control group was observed on days 12-20 of gestation in the 200 mg/kg group ( $\downarrow$  3.7% on day 12 and  $\downarrow$  4.6% on day 20). During the lactation period, there were no significant differences in the methyl salicylate groups as compared with the vehicle control group. During the gestation period, a significant depression in body weight gain as compared with the control group was observed on days 9-20 of gestation in the 200 mg/kg group (-4.08% on day 9; -36.2% on day 12; -22.8% on day 15; -20.4% on day 18 and -15.7% on day 20). During the lactation period, no significant differences in the methyl salicylate groups showed as compared with the vehicle control group.

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Table 2 Body weights in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight (g)											
Days of gestation	0	270.9±	12.0	(20)	270.6±	12.1	(20)	271.0±	10.1	(20)	270.8±	11.7	(20)
	3	292.3±	10.7	(20)	290.4±	13.1	(20)	291.1±	11.5	(20)	291.0±	10.1	(20)
	6	307.3±	10.2	(20)	303.6±	14.2	(20)	305.5±	13.0	(20)	305.0±	11.6	(20)
	9	320.3±	11.0	(20)	317.7±	16.2	(20)	320.5±	12.6	(20)	311.4±	13.6	(20)
	12	334.9±	12.9	(20)	331.2±	14.5	(20)	334.6±	14.2	(20)	322.6±	10.5*	(20)
	15	354.3±	14.6	(20)	349.8±	17.4	(20)	355.1±	15.9	(20)	341.3±	11.8*	(20)
	18	389.7±	16.7	(20)	386.7±	18.3	(20)	393.4±	20.6	(20)	370.6±	15.4**	(20)
	20	415.9±	17.0	(20)	414.4±	21.9	(20)	424.4±	23.7	(20)	396.6±	19.2*	(20)

\*: P<0.05, \*\*: P<0.01 (significantly different from vehicle control).  
Values are mean±S.D. and the values in parentheses represent the number of dams.

Table 2 - continued Body weights in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight (g)											
Days of lactation	0	331.4±	17.5	(20)	324.4±	20.5	(20)	333.9±	21.1	(20)	326.5±	15.8	(18)
	4	340.8±	12.6	(20)	334.6±	16.3	(20)	346.0±	18.1	(20)	331.6±	18.1	(18)
	7	345.1±	15.3	(20)	338.1±	16.6	(20)	348.9±	18.7	(20)	337.8±	14.8	(18)
	10	345.3±	14.4	(20)	342.5±	18.2	(20)	350.6±	17.0	(20)	344.1±	17.2	(18)
	14	350.0±	15.5	(20)	338.3±	18.3	(20)	349.0±	16.2	(20)	344.6±	16.6	(18)
	17	342.0±	13.8	(20)	335.0±	15.2	(20)	342.2±	15.7	(20)	341.2±	15.4	(18)
	21	330.6±	13.1	(20)	322.5±	14.6	(20)	326.7±	16.6	(20)	331.5±	11.4	(18)

Not significantly different from vehicle control.  
Values are mean±S.D. and the values in parentheses represent the number of dams.  
Two dams (200 mg/kg) died on day 23 of gestation.

Table 3 Body weight gains in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight gain (g)											
Days of gestation	9	13.0±	4.2	(20)	14.1±	5.7	(20)	15.0±	6.4	(20)	6.4±	6.6**	(20)
	12	27.6±	6.1	(20)	27.6±	4.6	(20)	29.1±	7.1	(20)	17.6±	7.3**	(20)
	15	47.0±	7.2	(20)	46.2±	8.3	(20)	49.5±	8.6	(20)	36.3±	7.1**	(20)
	18	82.4±	9.4	(20)	83.1±	10.5	(20)	87.9±	13.2	(20)	65.6±	11.3**	(20)
	20	108.6±	9.6	(20)	110.8±	14.3	(20)	118.9±	16.4	(20)	91.6±	15.4**	(20)

\*\* : P<0.01 (significantly different from vehicle control).  
Values are mean±S.D. and the values in parentheses represent the number of dams.

Table 3 - continued Body weight gains in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight gain (g)											
Days of lactation	4	9.4±	14.7	(20)	10.2±	15.2	(20)	12.1±	15.8	(20)	5.0±	11.1	(18)
	7	13.7±	13.6	(20)	13.7±	17.5	(20)	15.0±	15.7	(20)	11.3±	10.5	(18)
	10	13.9±	12.7	(20)	18.1±	18.0	(20)	16.7±	17.7	(20)	17.6±	12.1	(18)
	14	18.6±	16.4	(20)	13.9±	18.0	(20)	15.1±	15.3	(20)	18.1±	15.6	(18)
	17	10.6±	16.2	(20)	10.6±	16.6	(20)	8.3±	16.2	(20)	14.7±	14.2	(18)
	21	-0.8±	13.1	(20)	-1.9±	17.8	(20)	-7.2±	17.5	(20)	5.0±	15.2	(18)

Not significantly different from vehicle control.  
Values are mean±S.D. and the values in parentheses represent the number of dams.  
Two dams (200 mg/kg) died on day 23 of gestation.

- During the gestation period, a significant decrease in food consumption as compared with the control group was observed on day 9 of gestation in the 200 mg/kg group (-10.2%). During the lactation period, a significant decrease in food consumption as compared with the control group was observed on days 1-7 and 14-21 of lactation in the 200 mg/kg group (-42.9%, -15.4%, -16.3%, -11.8%, -10.3% and -21.9% on days 1, 4, 7, 14, 17 and 21 respectively).
- In the necropsy of the dams on day 22 of lactation, retention of oily fluid in the subcutis of the treated site was observed in all the dams in every group. In the necropsy of the dead dams in the 200 mg/kg group, retention of oily fluid in the subcutis of the treated site was observed in all the dams and dark red macule in the stomach were observed in 1 dam among them. Furthermore, 14 dead foetuses in the uterus were observed in 1 dam and craniorachischisis was observed in 2 dead foetuses among them.

Effects on offspring

- A significant decrease in the birth index (6%) and lower body weight (9.2%) in the males were observed in live newborns in the 200 mg/kg group as compared with the control group. A trend toward a decrease in the numbers of litter and live newborns and a trend toward an increase in the stillbirth index were also observed in the 200 mg/kg group. No significant difference in the sex ratio was observed in the methyl salicylate groups as compared with the control group. No abnormality was observed in the external examination of the live newborns in any group, but craniorachischisis was observed in 4 stillborns in the 200 mg/kg group and vestigial tail and anal atresia were observed in 1 stillborn in the 60 mg/kg group.

Table 7 Terminal delivery in Fo dams and F<sub>1</sub> offspring

Group and dose	Vehicle Control	20 mg/kg	60 mg/kg	200 mg/kg
No. of dams	20	20	20	20 g)
Gestational days a)	21.60 ± 0.50	21.90 ± 0.55	21.95 ± 0.22*	21.94 ± 0.42
No. of implantations b)	278(13.90 ± 2.00)	295(14.75 ± 1.41)	292(14.60 ± 2.06)	251(13.94 ± 2.13)
No. of litter b)	270(13.50 ± 2.16)	281(14.05 ± 1.57)	279(13.95 ± 2.50)	215(11.94 ± 3.33)
Gestation index c)	100	100	100	90.00
No. of live newborns b)	268(13.40 ± 2.09)	279(13.95 ± 1.47)	277(13.85 ± 2.52)	208(11.56 ± 3.36)
Birth index d)	96.40	94.58	94.86	82.87*
Sex ratio of live newborns e)	1.00(134/134)	0.94(135/144)	0.95(135/142)	0.94(101/107)
Body weight of live newborns (g) a)				
Male	6.5 ± 0.5	6.7 ± 0.6	6.6 ± 0.4	5.9 ± 0.6**
Female	6.0 ± 0.4	6.3 ± 0.6	6.3 ± 0.5	5.6 ± 0.7
No. of stillborns f)				
Male	0	2	0	2
Female	2	0	2	5
Total	2( 0.74)	2( 0.71)	2( 0.72)	7( 3.26)
No. of live newborns with external anomalies	0	0	0	0

\*: P<0.05, \*\*: P<0.01 (significantly different from vehicle control).

a) Values are mean±S.D.

b) Values in parentheses represent mean±S.D.

c) Values in represent percentages to the number of pregnant animals.

d) Values in represent percentages to the number of implantations.

e) Values in parentheses represent number of male/female live newborns.

f) Values in parentheses represent percentages to the number of litters.

Craniorachischisis was observed in 4 stillborns in the 200 mg/kg group and vestigial tail and anal atresia were observed in the 1 stillborn in the 60 mg/kg group.

g) Dams with live newborns were 18 among 20 dams.

- A trend toward a decrease (92.79%) in the viability index on day 4 was observed in the 200 mg/kg group as compared with the control group (98.13%) This change was not significant and was within the range of the background data (91.32 – 99.28%) of the institution. No significant differences in the weaning index were observed in the methyl salicylate groups as compared with the control group.
- Clinical observation after weaning: an abnormality of tooth, excessive elongation of the maxillary incisors, was observed beginning on day 24 after birth in 1 female in the 200 mg/kg group and this female died after showing hypoactivity, bradypnea and lateral position on day 31 after birth. An abnormality of tooth, excessive elongation of the maxillary incisors was also observed beginning on day 57 after birth in 2 males in the 200 mg/kg group. This change had recovered on day 87 after birth in 1 of these males but it continued until necropsy in the other male. Corectopia and dyscoria were observed beginning on day 51 after birth in 1 male and beginning on day 40 after birth in 1 female in the 200 mg/kg group, these changes continued until necropsy. Mass in the subcutis of the submacilla was additionally observed beginning on day 54 after birth in this male but this change had disappeared on day 88 after birth.
- A significant or a trend toward lower mean body weight as compared with the control group was observed throughout the lactation and maturation periods in the males and females in the 200 mg/kg group.

- A significant decrease in food consumption as compared with the control group was observed on days 28-63 after birth in the males and on days 28 and 70 after birth in the females in the 200 mg/kg group.
- A significant decrease in the differentiation indices of incisor eruption in the males and females, eyelid separation in the females and cleavage of the balanopreputial gland in the males were observed in the 200 mg/kg group as compared with the control group. No significant difference in the differentiation indices for pinna detachment, piliation, gait or descendus testis or vaginal opening was observed in the males or females in the methyl salicylate groups as compared with the control group.

Developmental Landmarks Parameter	Day of Acquisition (for 100% of the pups)			
	0	20	60	200
<b>Males</b>				
Balanopreputial Separation	45	47	61	72
Incisor Eruption	12	23	12	15
<b>Females</b>				
Eyelid Separation	15	16	15	17
Incisor Eruption	12	13	13	16

- No significant difference in the reflex indices for the righting reflex, ipsilateral flexor reflex, visual placing or preyer's reflex was observed in the males or females in the methyl salicylate groups as compared with the control group.
- Necropsy at weaning: A dilatation of the pelvic cavity was observed in 1 male in the 60 mg/kg group and 1, 2 and 1 females in the control, 20 and 60 mg/kg groups, respectively, and small testis was observed in 1 male in the 20 mg/kg group.
- Organ weight at weaning: In the males, a significant decrease in the absolute and relative weights of the liver and kidneys was observed in the 200 mg/kg group as compared with the control group. A significant decrease in the absolute weights of brain, adrenals and testes and a significant increase in the absolute weights of the brain, adrenals and testes and a significant increase in the relative weights of the brain and lungs were observed in the 200 mg/kg group as compared with the control group. In addition, a significant lower final body weight was observed in the 200 mg/kg group as compared with the control group. In the females a significant decrease in the absolute weights of the brain, heart, lungs, liver, kidneys, adrenals and ovaries and a significant increase in the relative weight of the brain were observed in the 200 mg/kg group as compared with the control group. In addition, a significant lower final body weight was observed in the 200 mg/kg group as compared with the control group.
- Skeletal examination at weaning: Skeletal anomalies were observed in 3 (3.90%), 6 (8.00%) and 20 (32.26%) offspring in the control, 60 and 200 mg/kg groups, respectively, and a significant increase in the incidence was observed in the 200 mg/kg group as compared with the control group. Considered by type of anomaly, nodulated rib was observed in 1 offspring in the 60 mg/kg group, fusion of the cervical vertebra was observed in 8 offspring (12.90%) in the 200 mg/kg group; fusion of the sternebra was observed in 3, 5 and 11 offspring in the control, 60 and 200 mg/kg groups, respectively; fusion of the thoracic vertebra was observed in 2 offspring in the 200 mg/kg group and misshapen sternebra was observed in 5 (8.06%) offspring in the 200 mg/kg group. Considering the incidences of these anomalies by type, a significant increase in the incidences of fusion of the cervical vertebra and misshapen sternebra was observed in the 200 mg/kg group as compared with the control group. Skeletal variations were observed in 20 (25.97%), 30 (40.00%) and 58 (93.55%) offspring in the control, 60 and 200 mg/kg groups, respectively, and a significant increase in their incidence was observed in the 200 mg/kg group as compared with the control group. Considered by type of variation, cervical rib was observed in 1 and 5 offspring in the control and 60 mg/kg groups,

respectively; short supernumerary rib was observed in 2 offspring in the 200 mg/kg groups; full supernumerary rib was observed in 45 (72.58%) offspring in the 200 mg/kg group; extra frontal ossification site was observed in 2 offspring in the 200 mg/kg group; accessory sternebra was observed in 11 (14.29%), 12 (16.00%) and 44 (70.97%) offspring in the control, 60 and 200 mg/kg groups, respectively; lumbarization was observed in 4 (6.45%) offspring in the 200 mg/kg group; shortened 13<sup>th</sup> rib was observed in 2 offspring in the control group; 7 lumbar vertebrae were observed in 39 (62.90%) offspring in the 200 mg/kg group; 8 lumbar vertebrae were observed in 1 (1.61%) offspring in the 200 mg/kg group; incomplete ossification of the cervical vertebra was observed in 1 (1.30%), 2 (2.67%) and 24 (38.71%) offspring in the control, 60 and 200 mg/kg groups, respectively; incomplete ossification of the thoracic vertebra was observed in 6 (7.79%), 10 (13.33%) and 43 (69.35%) offspring in the control, 60 and 200 mg/kg groups, respectively; incomplete ossification of the lumbar vertebra was observed in 22 (35.48%) offspring in the 200 mg/kg group; and incomplete ossification of the caudal vertebra was observed in 1, 4 and 3 offspring in the control, 60 and 200 mg/kg groups, respectively. Considering the incidences of these variations by type, a significant increase in the incidences of full supernumerary ribs, accessory sternebra, lumbarization, 7 lumbar vertebrae and incomplete ossification of the cervical, thoracic and lumbar centrum was observed in the 200 mg/kg group as compared with the control group.

Skeletal Examination in F <sub>1</sub> Offsprings			
	Dose (mg/kg/day)		
Parameter	0	60	200
№ litters examined skeletally	77	75	62
SKELETAL ANOMALIES			
№ of offsprings with skeletal anomalies (%)	3 (3.90)	6 (8.0)	20 (32.26)**
Nodulated ribs	0	1 (1.33)	0
Fusion of cervical vertebra	0	0	8 (12.90)**
Fusion of sternebra	3 (3.90)	5 (6.67)	11 (17.74)
Fusion of thoracic vertebra	0	0	2 (3.23)
Misshapen sternebra	0	0	5 (8.06)*
SKELETAL VARIATIONS			
№ of offsprings with skeletal variations (%)	20 (25.97)	30 (40)	58 (93.55)**
Cervical rib	1 (1.30)	5 (6.67)	0
Short supernumerary rib	0	0	2 (3.23)
Full supernumerary rib	0	0	45 (72.58)**
Extra frontal ossification site	0	0	2 (3.23)
Accessory sternebra	11 (14.29)	12 (16.0)	44 (70.97)**
Lumbarization	0	0	4 (6.45)*
Shortened 13 <sup>th</sup> rib	2 (2.60)	0	0
7 lumbar vertebrae	0	0	39 (62.90)**
8 lumbar vertebrae	0	0	1 (1.61)
Incomplete ossification of cervical vertebrae	1 (1.30)	2 (2.67)	24 (38.71)*
Incomplete ossification of thoracic vertebrae	6 (7.79)	10 (13.33)	43 (69.35)**
Incomplete ossification of lumbar vertebrae	0	0	22 (35.48)**
Incomplete ossification of caudal vertebrae	1 (1.30)	4 (5.33)	3 (4.84)

\*: p < 0.05, significantly different from control

\*\* : p < 0.15, significantly different from control

- Motor coordination: there was no significant difference in the number of falls in the males and females between the methyl salicylate groups and the control group.
- Learning ability: A significant shortening of the second swimming time was observed in the males in the 200 mg/kg group as compared with the control group. No similar change was observed in the first or third swimming times in the same group. No significant difference in the number of errors by the males or females and swimming time in the females was observed in the methyl salicylate groups as compared with the control group.
- Emotional behaviour: A significant decrease in the number of rearing (8.1) was observed in the females in the 200 mg/kg group as compared with the control group (12.6). The value was within the

range of the background data (6.0 – 8.7) of the institution and the value for the control group was higher than the background data. No significant differences in latency or the numbers of ambulation, defecation, urination or grooming in the males and females were observed in the methyl salicylate groups as compared with the control group.

- Reproductive ability: At the first mating, copulation was not confirmed in 1, 3 and 1 pairs in the control, 20, 60 and 200 mg/kg groups, respectively and 1 and 3 females in the 60 and 200 mg/kg groups respectively were sterile after copulation. The copulation indices were accordingly 95.00, 85.00, 95.00 and 93.75 % for the control, 20, 60 and 200 mg/kg groups, respectively and the male and female fertility indices were 100, 100, 94.74 and 80.00% for the control, 20, 60 and 200 mg/kg groups respectively, results showing no significant difference between the control and methyl salicylate groups. No significant difference was observed between the numbers of days required for copulation by the control and methyl salicylate groups. When 1, 3, 1 and 1 males in the control, 20, 60 and 200 mg/kg groups, respectively, in which copulation was not observed and 2 males in the 200 mg/kg group who had no paired female were mated with non-treated females, fertility was confirmed in all cases, excluding 1 male in the 200 mg/kg group for which copulation was not observed. When 1, 3, 1 and 1 females in the control, 20, 60 and 200 mg/kg groups, respectively, in which copulation was not observed were mated with males in the same group that were confirmed to have copulated, fertility was confirmed in all cases excluding 1 female in the 60 mg/kg group in which copulation was not observed.
- Body weight in F1 dams: a significant lower mean body weight as compared with the control group was observed on day 13 of gestation in the dams in the 200 mg/kg group.
- Necropsy of offspring: In the necropsy of the males after mating, excessive elongation of the maxillary incisors was observed in 1 male in the 200 mg/kg group and corectopia and dyscoria were observed in another male in the same group. In the necropsy of the females on day 13 of gestation, corectopia and dyscoria were observed in 1 female in the 200 mg/kg group. Dilatation of the pelvic cavity was observed in 1 female each in the control and 60 mg/kg groups and gritty material in the pelvic cavity was observed in 2 and 1 females in the control and 60 mg/kg groups, respectively. In the necropsy of non-pregnant and uncopulating females, no abnormality was observed in any group. In the necropsy of the dead female in the 200 mg/kg group, a small thymus was observed. As concerns excessive elongation of the maxillary incisors observed in this female in the clinical observation, the excessive elongation site was artificially cut before death.
- Cesarean section: There were no significant differences between the control and methyl salicylate groups in the numbers of corpora lutea, implantation or live embryos or in the pre-implantation losses and dead embryo index.