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Immune effects of respiratory exposure to fragrance chemicals

Pilot studies with isoeugenol and cinnamal

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Abstract

Effects of inhalation of fragrance chemicals on the immune system

Inhalation of the fragrance chemicals, isoeugenol and cinnamal, by mice resulted in immune reactions in the respiratory tract. This was observed in experiments performed by the RIVM (National Institute for Public Health and the Environment) of which results indicate that inhalation of some fragrance chemicals could induce unwanted effects on the immune system.

Fragrance chemicals are common ingredients in such consumer products as cosmetics and scented products. Several fragrance chemicals are known to cause allergy after skin exposure, but it is unknown whether inhalation of these fragrance chemicals can cause allergic reactions or other unwanted immune reactions. Till recently, it was assumed that inhalation of fragrance chemicals was harmless for humans, because there was no exposure via inhalation. However, applying fragrance chemicals in scented products used indoors, has changed this.

RIVM investigated the effects of inhalation of isoeugenol and cinnamal, fragrance chemicals that can cause skin allergy. Mice were exposed to the fragrance chemicals via inhalation. Effects on the immune system were measured using a respiratory lymph node assay, which measures cell proliferation in lymph nodes of the respiratory tract.

Inhalation of both isoeugenol and cinnamal resulted in stimulation of the immune system of the respiratory tract. The effects of isoeugenol were more pronounced than those of cinnamal. This is in contrast with results observed after skin exposure, after which both these fragrance chemicals were found equally potent in inducing skin allergy. This implies that effects of fragrance chemicals on the immune system depend on the route of exposure.

Relevant routes of exposure should then be used to predict the hazard of inhaling these compounds: skin for cosmetics and the respiratory tract for scented products. To obtain more insight into the hazards of fragrance chemicals used in scented products, RIVM is advising assessment of more fragrance chemicals in the respiratory lymph node assay.

Key words:

fragrance chemicals, respiratory exposure, immune effects, isoeugenol, cinnamal

Rapport in het kort

Effecten van inademing van geurstoffen op het immuunsysteem

Inademing van de geurstoffen isoeugenol en cinnamal leidt bij muizen tot een immuunreactie in de ademhalingswegen. Dat blijkt uit experimenten uitgevoerd door het RIVM. Deze resultaten geven aan dat inademing van sommige geurstoffen zou kunnen leiden tot ongewenste effecten op het immuunsysteem.

Geurstoffen komen voor in verschillende consumentenproducten, zoals cosmetica en geurproducten. Van verscheidene geurstoffen is bekend dat ze via de huid allergie kunnen veroorzaken, maar het is onbekend of ze ook allergische klachten of andere ongewenste immuunreacties kunnen veroorzaken via inademing. Tot nu toe is aangenomen dat inademing van geurstoffen niet schadelijk is voor de mens, omdat er geen blootstelling was via de ademhaling. De toepassing in geurproducten binnenshuis heeft hierin verandering gebracht.

Het RIVM onderzocht in experimenten de effecten van inademing van isoeugenol en cinnamal, geurstoffen die huidallergie kunnen veroorzaken. Muizen werden via inademing blootgesteld aan de geurstoffen. De effecten op het immuunsysteem werden gemeten met de respiratoire lymfkliertest, die celdeling als reactie meet in de lymfeklieren van de ademhalingswegen.

Inademing van zowel isoeugenol als cinnamal resulteerde in een stimulatie van het immuunsysteem van de ademhalingswegen. De effecten van isoeugenol waren sterker dan die van cinnamal. Dit is een verschil met blootstelling via de huid, waarbij beide geurstoffen eenzelfde potentie hebben om huidallergie te veroorzaken. Dit kan betekenen dat de effecten van geurstoffen op het immuunsysteem afhangen van de toedieningsroute.

Om het gevaar van inademing van deze stoffen te kunnen voorspellen, zal de relevante blootstellingsroute moeten worden gebruikt. Voor cosmetica is dat via de huid, voor geurproducten via inademing. Om meer inzicht te krijgen in de risico’s van geurstoffen in geurproducten, raadt het RIVM aan om meer geurstoffen te testen met de respiratoire lymfkliertest.

Trefwoorden:

geurstoffen, respiratoire blootstelling, immuuneffecten, isoeugenol, cinnamal

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Contents

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Summary

Fragrance chemicals are added as ingredients in several consumer's products, such as cosmetics and scented products. Some fragrance chemicals can elicit allergy after skin exposure and the dermal route is considered to be an important route of exposure. For a number of products such as cosmetics the respiratory exposure is not regarded as a relevant route of exposure, while for scented products the respiratory route is highly relevant. It is unknown if inhalation of fragrance chemicals that are known skin sensitizers can also induce allergy in the respiratory tract.

The effects of respiratory exposure to two known skin sensitizers, isoeugenol and cinnamal, have been investigated in a recently developed mouse model, the respiratory lymph node assay. This model is based on the local lymph node assay (LLNA), which is a validated model that is used to identify skin sensitizers. In the respiratory lymph node assay, BALB/c mice were exposed via inhalation, to either 300 mg/m^3 cinnamal or 300 mg/m^3 isoeugenol on three consecutive days for 45, 90, 180 and 360 minutes/day. Subsequently, the immune response was determined by measuring cell proliferation in the mandibular lymph nodes (LNs) that were found to be the most prominent reacting draining lymph nodes after inhalation exposure.

Both isoeugenol and cinnamal induced proliferation in the mandibular LNs. In the respiratory lymph node assay isoeugenol elicited clearly a higher immune response than cinnamal, as indicated by the cellular proliferation in the primary draining lymph node of the nasopharynx. This in contrast to data from the validated LLNA in which isoeugenol and cinnamal are equally potent. This implies that results from models using dermal exposure, such as the LLNA, can not be used to predict the hazard after respiratory exposure. To evaluate the hazard of fragrance chemicals that are used in scented products that may give rise to respiratory exposure, animal models that use the relevant route of exposure should be used. The respiratory lymph node assay appears to be a good model for this, but needs to be further validated with known skin and respiratory sensitizers and also with non-sensitizers and irritants. To further investigate the hazard of respiratory exposure to fragrance chemicals, more compounds should be evaluated in the respiratory lymph node assay.

1 Introduction

Fragrance chemicals are added as ingredients in several consumer's products, such as cosmetics, washing powders, and scented products to improve the smell in homes, offices, cars and stores. The last category contains many different products, for example bathroom sprays, incenses, fragrant candles, and room perfumes. Exposure to fragrance chemicals used in scented products is predominantly via inhalation. For some applications, such as room perfumes, this exposure is chronic, while for others, for instance bathroom sprays, exposure is now and then.

Skin exposure to certain fragrance chemicals can induce contact allergy. The EU Scientific Committee on Consumer and Non Food Products (SCCNFP) has compiled a list with 24 fragrance chemicals that are most frequently reported as contact allergens (22). For safety evaluation of fragrance chemicals the major route of exposure is considered to be the dermal route. Currently, exposure via inhalation is not regarded as a relevant route for toxic effects (8, 14), hence, in safety evaluations this route is not included. However, consumers can also be exposed via inhalation, when they use scented products that contain fragrance chemicals. It is not known if fragrance chemicals can induce respiratory allergy or other pulmonary immune reactions in healthy individuals, although in patients with asthma or chronic obstructive pulmonary disease (COPD), fragrance chemicals can provoke airway hyperreactivity and aggravate other clinical symptoms (13).

The Dutch Food and Consumer Product Safety Authority (VWA) has launched a project in 2006 in which the exposure and potential risks associated with the use of scented products is investigated (RIVM project 320105 'Ad hoc advice inspection product safety'). This project has given special attention to risk evaluation of fragrance chemicals that are known human skin sensitizers. Currently, no information is available to assess if these fragrances could be capable of sensitization via the respiratory tract (20, 21). There are only a few studies that have investigated effects of respiratory exposure to fragrance chemicals, and most of these studies do not include sensitization as an endpoint, but rather look at inhalatory toxicity or irritation. One of the recommendations made by the authors is that a protocol to quantify the respiratory sensitization potential should use the relevant route of exposure. Such a model could for instance be based on the murine Local Lymph Node Assay (LLNA), a model validated for the purpose to assess the skin sensitizing potential of chemicals, but in which exposure is via inhalation in order to assess the induction of respiratory immune responses.

It has to be considered that not only skin sensitizers, but respiratory sensitizers tested so far, were positive in the LLNA. Respiratory and skin sensitizers can be distinguished in the LLNA by the cytokine profile they induce. Skin sensitizers induce predominantly Th1 cytokines, whereas respiratory sensitizers induce Th2 cytokines (11, 26). With the current knowledge it is not known if potency determined in the LLNA is a good predictor for the potency of sensitization via the respiratory route. Therefore, LLNA data should be compared with data from experiments in which animals are exposed via the respiratory route. However, currently there are no validated models to assess respiratory sensitization (2).

Recently, TNO (Netherlands Organisation for Applied Scientific Research) together with the RIVM has developed a mouse model to assess the respiratory sensitizing potential of chemicals. In this model mice were exposed via the respiratory route on three consecutive days. The immune response was determined by measuring cell proliferation and cytokine responses in the lymph nodes draining the respiratory tract. In this model, the most pronounced effects were found in the mandibular lymph nodes, which drain the nasopharynx. In this respiratory lymph node assay, several known skin and respiratory sensitizers stimulate cell proliferation in the mandibular lymph nodes (1, 9, 25).

The respiratory lymph node assay has been used to investigate the hypothesis that inhalation of fragrance chemicals can induce immunostimulation after exposure via inhalation. This report describes the results of pilot studies which were conducted with two fragrance chemicals: isoeugenol and cinnamal. To expose mice to these fragrance chemicals two methods of distribution were used: vaporization with maximum vapour pressure or nebulization of aerosols in acetone. Isoeugenol and cinnamal are known human skin sensitizers (5, 6). A recent study on the frequency of sensitization to the 26 fragrances to be labelled according to the current EU legislation demonstrated that isoeugenol or cinnamal both can be regarded as important contact allergens (23). In addition, isoeugenol and cinnamal are classified as moderate sensitizers with reported EC3 values in the same range of 1.2-3.3% for isoeugenol (3, 4, 24) and 1.3% for cinnamal (12) .

2 Methods

2.1 Animals

Six to eight week old male BALB/c mice were obtained from the institute's own breeding colony. The animals were bred specific pathogen free (SPF) and kept in macrolon cages under conventional conditions. The mice were fed Hope Farms chow pellets (Woerden, the Netherlands) and water *ad libitum* during the whole experiment. The experimental setup of the study was examined and agreed upon by the institute's Ethical Committee on Experimental Animals, and all experiments were performed according to national legislation.

2.2 Fragrances

Isoeugenol (purity >99%) and cinnamal (purity >98%) were obtained from Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands.

2.3 Experimental design

In each experiment mice were randomly allocated to one of the seven experimental groups. In Table 1 test scheme and groups are presented. To obtain different dose groups mice were exposed for different periods of time to a fixed dose (concentration) of the fragrance chemicals. Mice were exposed nose-only to either isoeugenol or cinnamal for 45, 90, 180 or 360 minutes per day on day 0, 1 and 2. Controls were exposed to the vehicle for 360 minutes per day on day 0, 1, and 2. Mice were exposed to the fragrances either via evaporized or via nebulized test material, as described below.

Mice were placed in restraining tubes which were connected to one of the two central exposure chambers for nose-only exposure. Mice that were exposed to the vehicle control were connected to the exposure chamber for the vehicle and mice that were exposed isoeugenol or cinnamal were connected to the exposure chamber of the fragrance. Figure 1 illustrates the set-up for nose-only exposure.

In each experiment a dermal control was included. Mice were topically exposed to 10% isoeugenol in acetone: olive oil 4:1 (AOO) or 10% cinnamal in AOO on the dorsum of both ears (25 µl/ear) on day 0, 1 and 2. Control mice received the same treatment with the vehicle (AOO).

At day 5 mice were euthanized with nembutal and the auricular and mandibular lymph nodes (LN) were excised, pooled for each animal, and suspended in 5 ml RPMI 1640 (Gibco, Life Technologies, Breda, the Netherlands) with 5% heat inactivated Fetal Calf Serum (FCS) (Integro, Zaandam, the Netherlands), 100 units/ml penicillin and 100 µg/ml streptomycin (standard medium). At the autopsy other lymph nodes (deep cervical, parathymic, and mediastinal lymph nodes) were macroscopically examined for lymph node enlargement to indicate possible cellular stimulation.

Table 1 Experimental design

Group	*N=*	*Nose-only exposure Day 0, 1, 2*	*Duration (min/day)*	*Dermal exposure Day 0, 1, 2*
1	6	Vehicle control	360	None
2	6	Cinnamal or isoeugenol	45	None
3	6	Cinnamal or isoeugenol	90	None
4	6	Cinnamal or isoeugenol	180	None
5	6	Cinnamal or isoeugenol	360	None
6	6	None	--	Vehicle
7	6	None	--	Cinnamal or isoeugenol

2.4 Atmosphere generation and analysis

Isoeugenol and cinnamal were either vaporized or nebulized. In experiments were fragrances were vaporized, the maximum vapour pressure was used to vaporize the fragrances which is at 20^{o} C 11 ppm and 26 ppm for isoeugenol and cinnamal, respectively. These exposures are referred to as 'low concentrations'. The vapour in the exposure unit was sampled on activated charcoal at 190 ml/min for 15 minutes and used for wet chemical determination of the collected mass and used for to calculate the concentration of the test atmospheres.

In order to achieve higher concentrations than possible in the vapour phase, isoeugenol and cinnamal were nebulized in acetone to produce an aerosol of liquid droplets ('high concentrations'). The concentration of the solutions used for nebulization was 5 vol%, resulting in a concentration of 300 mg/m^3 when nebulized. In the 'high concentration' experiments isoeugenol and cinnamal aerosols were sampled on 47 mm Teflon filters at a flow rate of 1 litre/min for 5 minutes. The collected mass was determined gravimetrically immediately after sampling to minimize evaporations of the collected droplets and used for concentration calculations. The vapour in this mixture downstream of the filters was also sampled on activated charcoal. In addition, the test atmosphere was sampled at a flow rate of approx 1 litre/min for 5 minutes on activated charcoal and these were used for wet chemical determinations and used to calculate the average actual concentrations during the exposures.
The actual air concentrations measured were for isoeugenol and cinnamal are presented in Table 2.
The fluctuations of all test atmospheres were less than 10% as indicted by continuous mass concentration measurements using a Total Carbon Analyzer (TCA). For the high concentrations, the inlet of the TCA was heated to evaporate all droplets.

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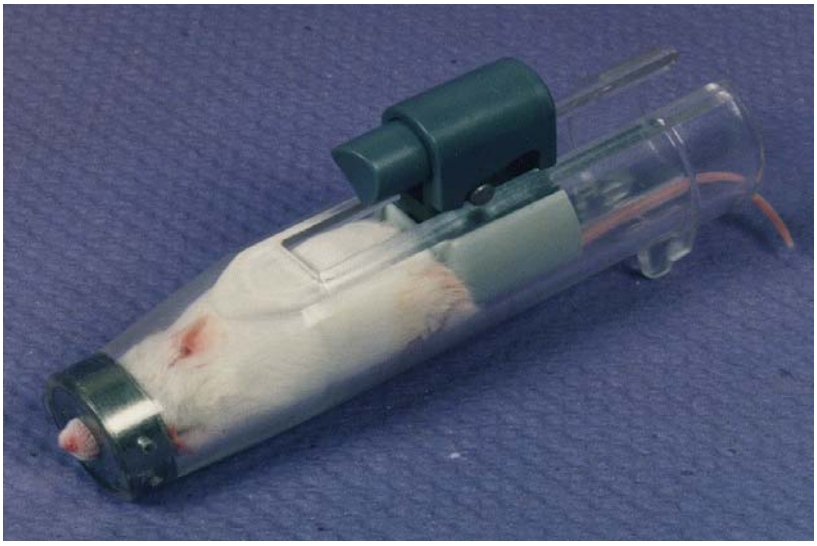

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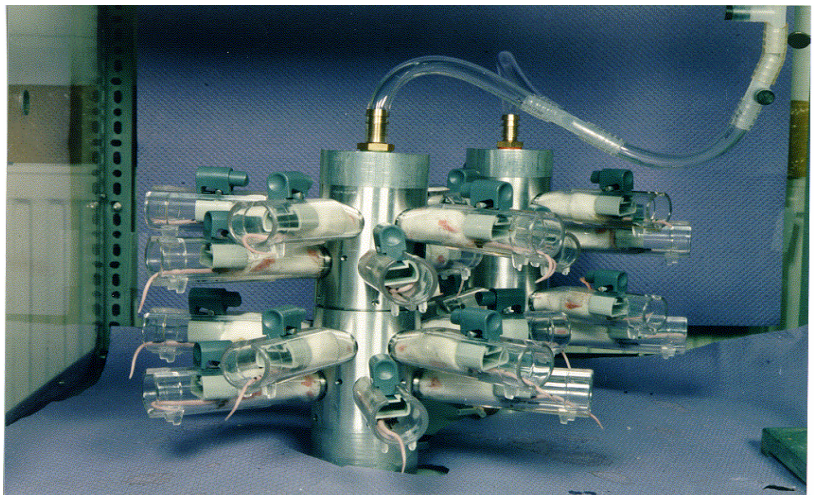

Figure 1 Experimental set-up for nose-only exposure. Mice are restraint in a tube (A) and are attached to the exposure chamber (B). Vehicle or fragrance were either vaporized by maximum vapour pressure or nebulized in acetone to produce an aerosol of liquid droplets

Table 2 Aerosol, vapour and total mass concentrations of 'high concentration' exposures

	Mass mg/m^3	Vapour mg/m^3	Total mg/m^3
Isoeugenol			
Day 1	253	75	328
Day 2	209	75	284
Day 3	-		
Cinnamal			
Day 1	145	143	288
Day 2	142	143	285
Day 3	166	143	309
Acetone		8360	
(both aldehydes)		(3500 ppm)	

2.5 Assessment of cell proliferation

Single cell suspensions were prepared in standard medium with 5% FCS under aseptic conditions by pressing the auricular and mandibular LN trough a 70 µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed in standard medium with 5% FCS (10 minutes, 300 g, 4 °C) and resuspended in 1 ml standard medium with 10% FCS. A Coulter Counter (Z2, Coulter Electronics, Mijdrecht, the Netherlands) was used to count the cells. Then the concentration of the cell suspensions was adjusted to 1×10^7 cells/ml.

Of each cell suspension, 200 µl was seeded in triplicate in a U-bottom 96-well tissue culture plate (Greiner, Alphen aan den Rijn, the Netherlands). After addition of 10 µl/well (37 kBq methyl-3H-thymidine (specific activity 185 MBq/mmol, Amersham Biosciences, Buckinghamshire, UK) the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 during 20–24 h. The cells were harvested on glass-fiber filters (LKB-Wallac, Turku, Finland) using a multiple cell culture harvester (LKB-Wallac). The [^{3}H]-thymidine activity was determined using a liquid scintillation counter (1205 Betaplate TM, LKB-Wallac). For further calculations the median of the triplicates was used. The [^{3}H]-thymidine incorporation is expressed per animal, being the measured counts per minute (cpm) times the cell number of the two LN and divided by the cell number in culture. The mean [^{3}H]-thymidine incorporation per experimental group ± SEM was calculated. Stimulation indices (SI) were calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the vehicle group. The SI after respiratory exposure was calculated by using the nose-only vehicle group and the SI after dermal exposure by using the dermal vehicle group.

2.6 Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Significant differences of the control group were determined with the Bonferroni post hoc test, using a significance level of p=0.05.

3 Results

3.1 Effects of exposure to isoeugenol via maximum vapour pressure

Exposure to isoeugenol (11 ppm) increased cell number and cell proliferation in the mandibular LNs (Table 3). Figure 2 shows the SIs of the mandibular LNs. The increase in cell proliferation did not show a time-dependent effect and the response was highly variable. None of the observed effects were statistically significant.

Exposure to isoeugenol did not increase proliferation in the auricular LNs. However, the cell number in the control group was higher than normally observed. Therefore, the calculated SIs are below 1. Dermal application of 10% isoeugenol resulted in a SI of the auricular LNs of 29.3 (Table 3).

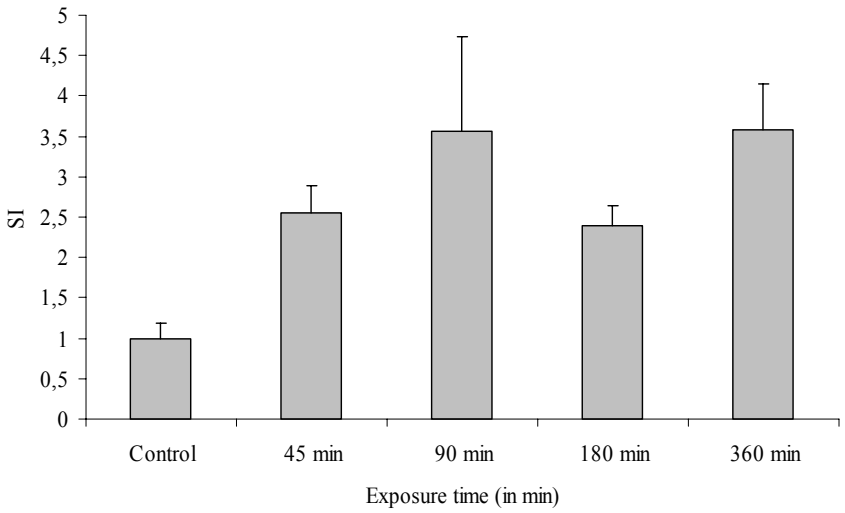

Figure 2 Stimulation index of the mandibular LNs after nose-only exposure to 11 ppm isoeugenol via vaporization. Stimulation indices are shown as mean ± SEM (n=6 mice per group).

Table 3 Effects of isoeugenol on mandibular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Control	2.81 ± 1.44	1232 ± 596	1.0 ± 0,48
45 min/day	4.57 ± 1.49	3150 ± 1004	2.56 ± 0.81
90 min/day	5.69 ± 2.79	4383 ± 3530	3.56 ± 2.86
180 min/day	5.37 ± 1.20	2952 ± 711	2.40 ± 0.58
360 min/day	7.03 ± 2.19	4815 ± 1575	3.58 ± 1.40

Results are shown as mean ± SEM (n=6 per group). Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group.

Table 4 Effects of isoeugenol on auricular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Inhalatory exposure			
Control	6.46 ± 1.82	4406 ± 1766	1 ± 0.4
45 min/day	3.70 ± 1.34	1531 ± 414	0.35 ± 0.09
90 min/day	3.95 ± 0.98	1640 ± 477	0.37 ± 0.011
180 min/day	4.64 ± 1.35	2068 ± 919	0.47 ± 0.21
360 min/day	4.41 ± 1.63	1955 ± 584	0.43 ± 0.13
Dermal exposure			
Control	3.92 ± 2.00	1278 ± 221	1.0 ± 0.17
10% Isoeugenol	23.7 ± 6.82	37477 ± 13144	29.3 ± 16.7

Results are shown as mean ± SEM (n=6 per group). Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. The SI after respiratory exposure was calculated by using the nose-only vehicle group and the SI after dermal exposure by using the dermal vehicle group.

3.2 Effects of exposure to cinnamal via maximum vapor pressure

Exposure to cinnamal (26 ppm) slightly increased cell number, cell proliferation and SIs in the mandibular LNs (Table 5, Figure 3). The variance in the experimental groups was high and none of the observed effects were statistically significant.

Cinnamal did not affect proliferation in the auricular LNs, except for mice that were exposed for 360 min/day. In this group the proliferation was 1.8 fold higher than in the control group. Ear application of 10% cinnamal resulted in a SI of 10.1 (Table 6).

Table 5 Effects of cinnamal on mandibular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Control	2.69 ± 0.55	741 ± 209	1.0 ± 0.28
45 min/day	2.97 ± 1.15	1103 ± 417	1.49 ± 0.56
90 min/day	3.43 ± 0.52	1870 ± 954	2.52 ± 1.29
180 min/day	4.56 ± 1.37	1823 ± 766	2.46 ± 1.03
360 min/day	4.62 ± 0.62	1700 ± 345	2.53 ± 0.71

Results are shown as mean ± SEM (n=6 per group). Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group

Table 6 Effects of cinnamal on auricular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Inhalatory exposure			
Control	4.28 ± 2.05	1536 ± 685	1.0 ± 0.45
45 min/day	4.05 ± 0.32	1319 ± 214	0.86 ± 0.14
90 min/day	4.22 ± 0.68	1440 ± 251	0.94 ± 0.16
180 min/day	4.53 ± 0.96	1332 ± 748	0.87 ± 0.49
360 min/day	6.88 ± 2.14	2765 ± 685	1.80 ± 0.50
Dermal exposure			
Control	4.04 ± 0.52	1243 ± 685	1.0 ± 0.13
10% Isoeugenol	13.4 ± 1.54	12532 ± 2885	10.1 ± 2.32

Results are shown as mean ± SEM (n=6 per group). Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. The SI after respiratory exposure was calculated by using the nose-only vehicle group and the SI after dermal exposure by using the dermal vehicle group.

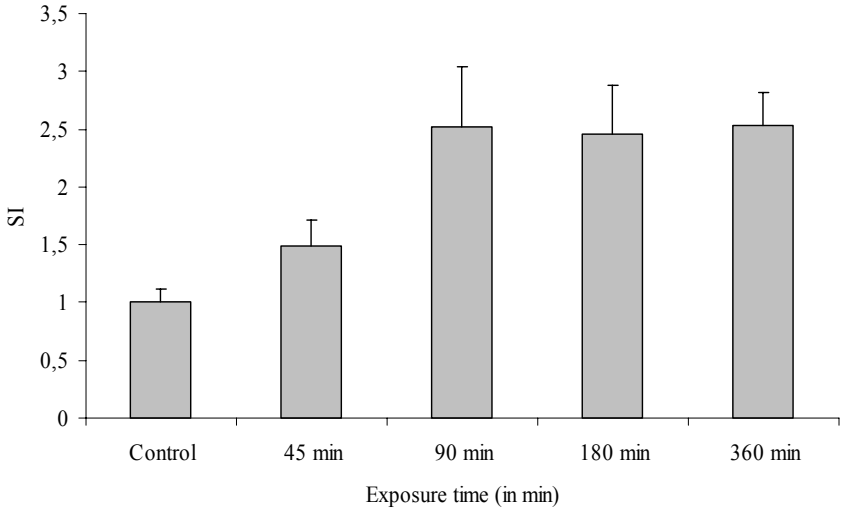

Figure 3 Stimulation index of the mandibular LNs after nose-only exposure to 26 ppm cinnamal via vaporization. Stimulation indices are shown as mean ± SEM (n=6 mice per group).

3.3 Effects of exposure to aerosols of isoeugenol

Exposure to aerosols of isoeugenol (300 ppm) resulted in toxic effects in the mice that were exposed for 360 min/day. After two days of exposure one mouse died and the other mice displayed several signs of distress. These mice were not exposed to isoeugenol on the third day. Effects of the two days exposure to isoeugenol were assessed on day 5. On the third day two mice died that were exposed for 180 minutes/day for 3 days. The other mice in this group appeared normal.

Exposure to isoeugenol aerosols resulted in a significant increase of cell number and cell proliferation in the mandibular LNs (Table 7, Figure 4A). This increase was time-dependent, with the exception of the group that was exposed for 360 minutes/day. This group, however, was exposed for two days only. Effects of isoeugenol on cell number and cell proliferation were statistically significant for all exposure groups.

Isoeugenol exposure for 90 minutes/day or longer increased proliferation in the auricular LNs (Table 8, Figure 4B). The mean SI in the auricular LNs was a factor 3-4 higher than in the mandibular LNs, but the variance was very high. Dermal exposure to 10% isoeugenol resulted in a SI of 18.8 (Table 8).

Table 7 Effects of isoeugenol on mandibular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Control	2.34 ± 0.57	1070 ± 325	1.0 ± 0.30
45 min/day	5.18 ± 0.77**	4329 ± 688*	4.04 ± 0.64*
90 min/day	6.06 ± 1.41***	5486 ± 2515**	5.13 ± 2.35**
180 min/day[a]	5.14 ± 1.32**	6555 ± 2423***	6.13 ± 2.26***
360 min/day[b]	5.54 ± 1.19**	4864 ± 1532*	4.54 ± 1.43*

Results are shown as mean ± SEM (n=6 per group). [a] n=4; exposure for 3 days; [b] n=5, exposure for 2 days. Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 8 Effects of isoeugenol on auricular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Inhalatory exposure			
Control	3.38 ± 0.50	1493 ± 229	1 ± 0,15
45 min/day	4.74 ± 1.06	2528 ± 863	1.69 ± 0.58
90 min/day	10.7 ± 7.18	29549 ± 39947	19.79 ± 26.76
180 min/day[a]	10.7 ± 4.06	33648 ± 38004	22.54 ± 25.46
360 min/day[b]	8.1 ± 3.12	15581 ± 15115	10.44 ± 10.12
Dermal exposure			
Control	3.31 ±0.87	1698 ± 472	1.0 ± 0.28
10% Cinnamal	20.0 ±5.01	31941 ± 21910	18.8 ± 8.3

Results are shown as mean ± SEM (n=6 per group). [a] n=4; exposure for 3 days; [b] n=5, exposure for 2 days. Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. The SI after respiratory exposure was calculated by using the nose-only vehicle group and the SI after dermal exposure by using the dermal vehicle group.

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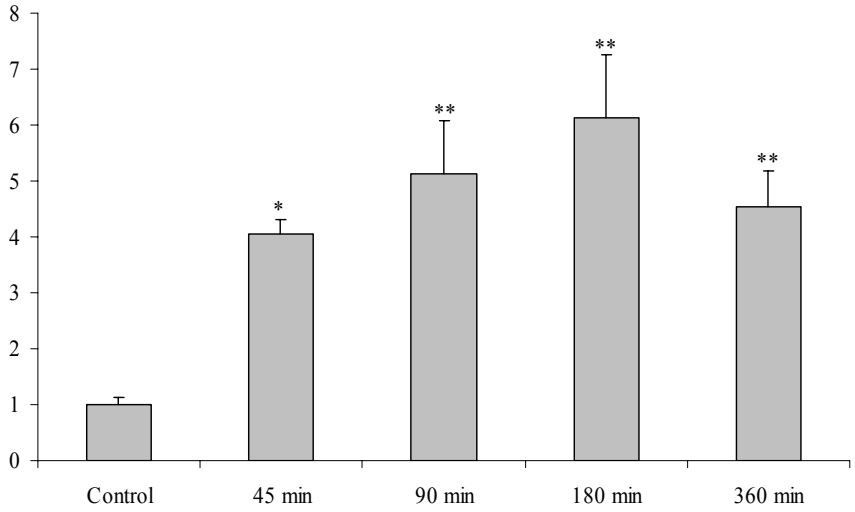

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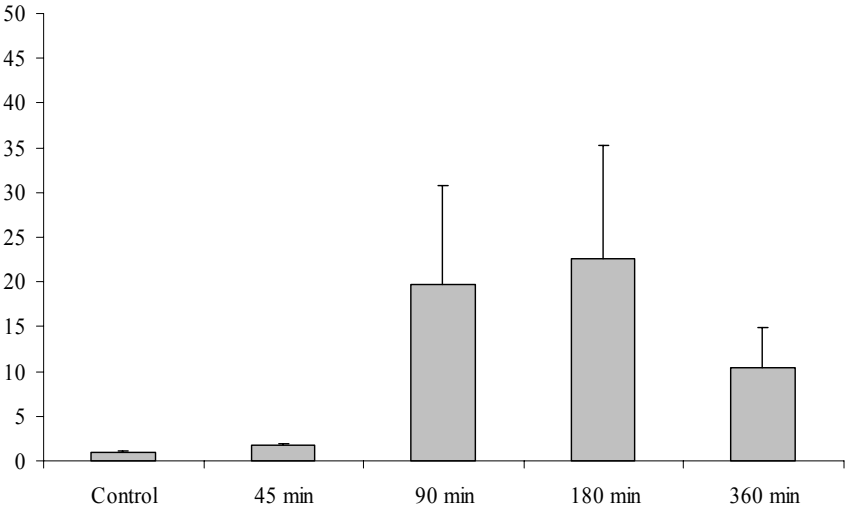

Figure 4 Stimulation index of the mandibular LNs (A) and auricular LNs (B) after nose-only exposure to 300 ppm isoeugenol via nebulization of aerosols in acetone. Stimulation indices are shown as mean ± SEM (n=6 mice per group exposure, except for the 180 minutes group: n=4 and the 360 minutes group: n=5). Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.4 Effects of exposure to aerosols of cinnamal

Toxic effects were also observed after nose-only exposure to aerosols of cinnamal (300 ppm). Two mice died after exposure to cinnamal for 360 minutes/day for 1 day. The other mice in this group displayed several signs of distress. These mice were not exposed to linnamal on day 2 and 3. Effects of one day exposure to linnamal were assessed on day 5.
Exposure to cinnamal aerosols increased cell number significantly in mice that were exposed for 180 minutes/day. Cell proliferation and SIs were significantly increased in mice that were exposed for 90 minutes/day and for 180 minutes/day (Table 9, Figure 5).
Cinnamal exposure for 180 minutes/day also increased proliferation in the auricular LNs. However, not all mice in the group responded, the proliferation rates were highly variable. Ear application of 10% cinnamal resulted in a SI of 17.8 (Table 10).

Table 9 Effects of cinnamal on mandibular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Control	2.81 ± 0.63	1243 ± 392	1.0 ± 0,32
45 min/day	3.33 ± 0.49	1705 ± 424	1.37 ± 0.34
90 min/day	3.86 ± 0.62	2168 ± 368*	1.74 ± 0.30*
180 min/day	4.35 ± 0.90*	2489 ± 598**	2.00 ± 0.48**
360 min/day[a]	2.97 ± 1.50	1514 ± 775	1.22 ± 0.62

Results are shown as mean ± SEM (n=6 per group).). [a] n=4; exposure for 1 day. Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * $p<0.05$, ** $p<0.01$.

Table 10 Effects of cinnamal on auricular LNs: cell number, cell proliferation and SIs

Group	Cell number	Proliferation	Stimulation index
Inhalatory exposure			
Control	3.41 ± 0.73	1603 ± 362	1 ± 0,2
45 min/day	3.83 ± 0.67	1424 ± 254	0.89 ± 0.16
90 min/day	4.67 ± 0.54	2266 ± 471	1.41 ± 0.29
180 min/day	8.31 ± 4.85	5275 ± 4272	3.29 ± 2.67
360 min/day[a]	5.73 ± 2.69	3184 ± 1001	1.99 ± 0.62
Dermal exposure			
Control	3.77 ± 0.73	1611 ± 314	1.0 ± 0.20
10% Cinnamal	14.6 ± 5.01	28617 ± 11427	17.8 ± 11.9

Results are shown as mean ± SEM (n=6 per group). [a] n=4; exposure for 1 day. Cell number is expressed as 10^6 cells, proliferation is expressed as cpm per mouse. SIs are calculated by dividing the [3H]-thymidine incorporation of the experimental group with the mean [3H]-thymidine incorporation of the control group. The SI after respiratory exposure was calculated by using the nose-only vehicle group and the SI after dermal exposure by using the dermal vehicle group.

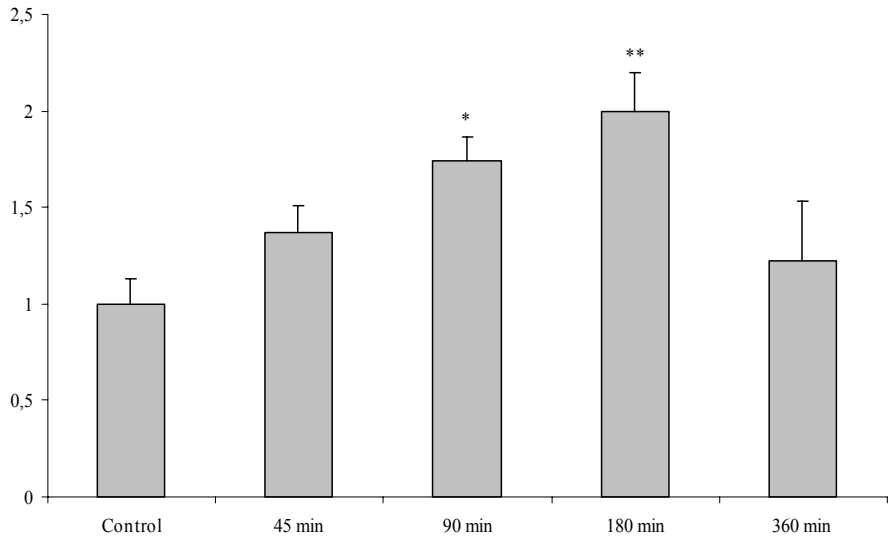

Figure 5 Stimulation index of the mandibular LNs after nose-only exposure to 300 ppm cinnamal via nebulization of aerosols in acetone. Stimulation indices are shown as mean ± SEM (n=6 mice per group, except for the 360 minutes group: n=4). Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * p<0.05, ** p<0.01.

4 Discussion

The fragrance chemicals, isoeugenol and cinnamal, two known skin sensitizers, have been tested in the respiratory lymph node assay to assess their effects on the immune system after respiratory exposure.

The chemicals were distributed either via vaporization using maximum vapour pressure or via nebulization of the chemicals in acetone. The way of distribution clearly influenced the exposure concentration. With maximum vapour pressure maximum exposure levels of 11 and 26 ppm can be reached for isoeugenol and cinnamal, respectively. Exposure to these concentrations resulted in a slight effect on cell proliferation in the mandibular LNs, but this effects was highly variable. To obtain information on dose-response relationships, total exposure dose was increased by increasing exposure time to a fixed concentration. Cinnamal and isoeugenol did not induce dose-dependent effects. The substance is delivered as a mixture of vapour and liquid droplets that will at least lead to local high dose levels due to impaction of the pure substance on respiratory tract epithelium. The size of the droplets (~ 5 μm) prevents them to reach the lower airways and alveoli, though once deposited, the compound can continue to evaporate resulting in significantly higher concentrations in the alveolar air spaces.

The exposure concentration was increased, by exposing the mice via nebulization of the fragrance chemicals dissolved in acetone. With this approach, aerosols are generated that are approximately 1-2 μm and these aerosols will deposit predominately in the nasopharyngeal area (18). Based on data from these two fragrance chemicals, exposure via nebulization is the preferred way in this respiratory model, because with this approach higher exposure concentrations can be used. This way of exposure is also similar to the method used in the respiratory lymph node assay as presented previously (1, 9). Both fragrance chemicals increased also proliferation in the auricular LNs. The effects were most pronounced after isoeugenol exposure and occurred after exposure for 90 minutes or more. The effects were quite heterogeneous, in some mice very high SIs were observed, while in other mice hardly any increased cell proliferation was observed. This phenomenon has also been reported for some skin and respiratory sensitizers in the respiratory LLNA (unpublished data) and is probably the consequence of deposition of the aerosols on the skin in the nose area and subsequent absorption through the skin and activation of the draining (auricular) LN.

The potency of isoeugenol and cinnamal has been established in the LLNA and both are classified as moderate skin sensitizers with reported EC3 values in the range of 1.2-3.3% for isoeugenol (3, 4, 24) and 1.3% for cinnamal (12). Remarkably, in the respiratory lymph node assay the proliferative response in the mandibular LN was different for these two fragrance chemicals. After exposure to isoeugenol aerosols a significant time (dose) dependent increase of cellular proliferation was observed, for mice exposed for 45 minutes/day or longer. In contrast, although cinnamal exposure did result in a statistically significant increase of cell proliferation in the mandibular LNs, after exposure for 90 or 180 minutes/day, cell proliferation was a factor 3 lower compared to isoeugenol. Hence, respiratory exposure to isoeugenol and cinnamal, which are equally potent after dermal exposure, elicits a different immune stimulation in the mandibular LNs.
Previously, isoeugenol was tested in the mouse IgE test, an approach that is used to classify respiratory sensitizers. The chemicals are applied on the skin and it is thought that only respiratory sensitizers induce IgE (17). In this approach, isoeugenol was negative and was considered lack the potential to be a respiratory sensitizer (16). Although we did not investigated the induction of IgE, our results show that isoeugenol can induce immune responses in the respiratory tract. The immune effects in this model were found predominantly in the mandibular LNs which is probably caused by the deposition of the

aerosols in the nasopharyngeal area. These results do not indicate that these fragrance chemicals can induce respiratory allergy. However, immune reactions caused by chemicals in the lung can also induce other pulmonary reactions. Some chemicals induce a Th1-type immune responses in the lungs, as has been shown in rodent models. Skin allergens such as DNCB (dinitro-chloro-benzene), DNFB (dinitro-fluoro-benzene) and picryl chloride were able to induce allergic reactions in the lungs, e.g. laryngitis, pneumotis, and airway hyperreactivity to non-specific stimuli. All these immune reactions were independent of IgE (2, 7, 15). The cytokine profiles that are induced by isoeugenol and cinnamal in the LLNA and in the respiratory lymph node assay could provide more insight in the possible immune reactions that could occur after inhalation. Dermal exposure to cinnamal induced an increase of Th1 cytokines (19), for isoeugenol no such data are publicly available. To further investigate the type of immune response these fragrance chemicals induce, and the pathological consequences of these responses, assessment of cytokine profiles after both dermal and respiratory exposure to isoeugenol and cinnamal, in materials collected in the described experiments, need to be performed. In addition, lung function parameters should be assessed.

It is, however, to early to designate these fragrances as respiratory sensitizers. The respiratory lymph node assay is a recently developed animal model and more research is needed to further validate this model. In the LLNA a cut-off value of SI=3 is used to discriminate skin sensitizers from non-sensitizers and irritants (10). With the current knowledge, a cut-off value is not available for the respiratory lymph node assay. The distribution of chemicals in the respiratory tract is different from distribution in the skin. Furthermore, the immune reaction elicited in the skin can mechanistically be different from the immune reaction induced in the lungs. More research is needed in order to choose the appropriate cut-off point in the respiratory lymph node assay. Some known skin and respiratory sensitizers have already been tested in this model and these preliminary data show that potency ranking is slightly different from the LLNA (1, 9). Additional experiments with other skin and respiratory sensitizers, but also irritants should be performed to get more insight in the effects of the route of exposure on the immune reactions of these chemicals in order to validate the respiratory lymph node assay and to decide if this approach can be used to predict the effects on the immune system after inhalation of chemicals .

In conclusion, these pilot studies have shown that effects of fragrance chemicals that have a similar potency in the LLNA, induce different immune responses in the respiratory local lymph node assay. To further investigate the hazard of fragrance chemicals, more fragrance chemicals of the SCCNFP list should be investigated in this model.

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