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Decreased viability and proliferation of Chang conjunctival epithelial cells after contact with ultraviolet light-irradiated pollen.

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Decreased viability and proliferation of CHANG conjunctival epithelial cells after contact with ultraviolet light-irradiated pollen.

ABSTRACT

Context - Contact with pollen is the major reason for the development of allergic symptoms on the ocular surface leading to a significant increase of allergic diseases worldwide.

Environmental changes such as increased ultraviolet (UV) radiation and air pollution are discussed as contributory causes for this increase.

Objective – We investigated the effect of ultraviolet (UV) light on the histamine content of pollen and examined if an irradiation of pollen affects the viability and proliferation of conjunctival cells.

Materials and Methods - Alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) pollen were irradiated for different time periods with sunlight, UV-A or UV-B light and the histamine content was analysed and compared with non-irradiated pollen. Conjunctival epithelial cells (CHANG cells) were exposed to irradiated and non-irradiated pollen followed by an assessment of cell viability with the colorimetric MTS test and the impedance based measurement of cell proliferation using the xCELLigence real time analysis system.

Results - UV light irradiation increased the histamine level of alder and hazelnut pollen in a dose dependent manner. CHANG cells treated with irradiated pollen induced a statistically significant higher decrease of cell viability than treatment with non-irradiated pollen.

Discussion and conclusion - Our results indicate that UV light is able to alter pollen thus making them more harmful for conjunctival cells.

Key words: cell viability, conjunctival cells, pollen, ultraviolet light, histamine

Introduction

Pollen are known for being the major source for the development of allergic diseases such as allergic rhinitis, allergic conjunctivitis or allergic asthma (1–3). In the last years a significant rise of allergies occurred worldwide. Some of the major reasons are thought to be the increasing air pollution and the increased level of ultraviolet (UV) light reaching the earth's surface. (4–6). It is assumed that pollutants can modify or alter the allergenic potential of pollen and thus reinforce allergic symptoms (7). Pollutants can accumulate on the pollen surface and lead to visible and/or functional alterations which could exacerbate negative effects on human health (8–10). Studies indicate that humans living in polluted urban areas are more likely to develop allergic diseases and asthma than humans who live in rural areas (11–13).

Through climate change higher levels of harmful ultraviolet (UV) light reach the earth's surface which causes a premature start of the pollen season and an extension of the length of the pollination period (14–16). Pollen are in direct contact with sunlight for hours, days or often weeks until they find their way to human eyes, the nose or the respiratory tract where they are able to provoke allergic symptoms.

It is known that UV light has an influence on the pollen germination, tube growth and also on the viability of pollen (17,18). It seems feasible that UV light may also induce alterations of pollen ingredients during this time period.

In this study the effect of UV light on the histamine content of pollen and the impact of irradiated and non-irradiated pollen on viability and proliferation of human conjunctival cells were investigated.

Material and Methods

Pollen collection and irradiation

We collected pollen grains of the common tree species black alder (*Alnus glutinosa* (L.) Gaertn.) as well as hazel (*Corylus avellana* L.) in the local area (south and south-east of Styria) in 2017. Male inflorescences were harvested, sieved and stored at room temperature in paper bags protected from light until analysis. Shortly before use pollen were suspended in physiological saline in concentrations from 10 to 100 mg/ml and then irradiated with UV-A light, UV-B light or sunlight. To avoid evaporation due to irradiation all solutions were irradiated in sealable, UV light permeable fused silica vessels. Irradiation with UV-A or UV-B light was done for different time periods. For irradiation an UV-A lamp (VL-115L, 365 nm Tube, Power: 30 W) and UV-B lamp (VL-115 M, 312 nm Tube, Power: 30 W) from Vilber-Lourmat GmbH, France, were used. The irradiance was measured with an Optometer P 9710 from Gigahertz Optik GmbH, Germany, and amounted to 45 W/m² for UV-A and 43 W/m² for UV-B light. The irradiance we used corresponded to natural occurring irradiance for UV-A light. For UV-B light the irradiance was approximately 40 times higher than the natural occurrence in Austria.

In addition, sunlight was used for irradiation as well. Therefore, the fused silica vessels were placed on the roof terrace of our department for one or two days. Experiments were carried out in April 2017 and the irradiance of UV light was measured with an Optometer P 9710 at three points of time 10 am, 12 am, 2 pm and amounted to 23 W/m², 43 W/m² and 30 W/m² for UV-A and 1.1 W/m², 1.7 W/m² and 1.1 W/m² respectively for UV-B light. Considering sunrise and sunset the average sunshine duration amounted to 10 hours per day. Together with the above mentioned time-dependent differences in the irradiance the mean irradiance with sunlight could be estimated with 32 W/m² for UV-A and 1.3 W/m² for UV-B light (19).

Determination of histamine content

For histamine measurements the commercially available ELISA kits from Creative Diagnostics (Histamine Research ELISA, Cat. No. DEIA207) and Immusmol (Histamine ELISA Kit, Ref. BA-E 5800) were used following the protocols recommended by the manufacturers. Pollen were used in a concentration of 100 mg/ml suspended in physiological saline shortly before use. The solutions were then irradiated with sunlight for one day and UV-A and UV-B light for 2, 4 or 6 hours. Absorptions were read out with the Flex-Station 3 Multi-Mode Microplate Reader set (Molecular Device) at a wavelength of 450 nm and a reference wavelength of 620 nm. The calibrator curve was obtained by plotting the absorbance readings (mean absorbance) of the standards linear on the y-axis against the corresponding standard concentrations logarithmic on the x-axis using a 4-parameter non-linear regression for curve fitting. The experiments were carried out using both test kits and all samples were analysed in duplicates.

Assessment of cell viability

Human conjunctival cells (CHANG cells, CCL-20.2, clone 1-5c-4, Wong-Kilbourne derivative of CHANG conjunctiva) acquired from the American Type Culture Collection were cultivated (ATCC, Manassas, Va., USA). Cells were grown in DMEM (Dulbeccos Modified Eagle Medium - Gibco™, Thermo Fisher Scientific) and 1 % P/S (penicillin/streptomycin, Biochrom, Germany) and 10 % fetal bovine serum (GE Healthcare Bio-Sciences GmbH, Austria) in a CO₂ incubator (Heracell 240, Kendro Heraeus, Germany) at 37°C, 5 % CO₂. The cells were used when at least 90 % confluent, assessed by an inverse microscope (Axio Observer Z.1, Zeiss, Germany).

For the experiments cells were seeded in 96-well plates (Becton Dickinson GmbH, Austria) in a concentration of 100.000 cells per ml and incubated overnight. On the following day the medium was removed and the cells were rinsed twice with DPBS (Dulbecco's Phosphate-Buffered Saline - Gibco™, Thermo Fisher Scientific, Austria). Afterwards the wells were filled with 100 µl of test solutions: previous experiments revealed that alder pollen are much more harmful than hazel pollen. We tested several pollen concentrations, finally a concentration of 10 mg/ml for alder and 25 mg/ml for hazel pollen tended to be the optimal concentrations to study the effects on conjunctival cells. Pollen were suspended in physiological saline and irradiated for 24 hours with UV-A and UV-B light before the experiment. We analysed pollen suspensions as well as pollen supernatants; therefore, the suspensions were centrifuged for 5 minutes at 1300 rpm and the supernatants were collected for further use. Plates were then incubated for 30 minutes at 37 °C in a CO₂ incubator. Afterwards suspensions and supernatants were removed and wells were carefully washed two times with DPBS to remove all pollen and then filled with 100 µl DMEM. For the determination of cell viability, the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS test) from Promega, USA, was used. The MTS assay is a colorimetric method for the determination of the number of viable cells in proliferation, and it contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] which is bio reduced by cells into a coloured formazan product. As the bio reduction only takes place in metabolically active cells, results can be directly related to the number of viable cells. 10 µl of MTS reagent were added to the medium in all wells and incubated for two hours in the incubator. The absorbance of the MTS reaction product was measured using an ELISA reader (Anthos 2010, Anthos Labtec Instruments GmbH, Germany) at a wavelength of 492 nm and a reference wavelength of 620 nm.

Each experiment was repeated three times, and for the calculation of the cell viability of the samples the mean of seven wells per assay was used. Cells treated with culture medium served as control and cell viability was assumed with 100 %.

Assessment of cell proliferation

We assessed the proliferation of CHANG cells after contact with pollen using the impedance-based xCELLigence Real-Time Cell Analysis (RTCA) system (ACEA Biosciences, San Diego, CA). For the background reading, 100 µl of cell culture media were added to each of the 16 wells of the E-Plate (distributed by Omni Life Sciences, Bremen). Afterwards, 100µl of cell suspension containing 10.000 cells were added to each well. The cells have been incubated for 24 hours on the xCELLigence station inside the incubator, and changes in impedance reflecting cell adhesion and proliferation were measured every 20 minutes. On the following day the media was removed, and all wells were washed once with DPBS and then filled with 100 µl of the test solutions:

Alder and hazel pollen were prepared at concentrations of 20 mg/ml and 50 mg/ml respectively in physiological saline, irradiated with UV-A light, UV-B light and sunlight and then centrifuged for 5 min. at 1300 rpm. The supernatant was diluted with DMEM at a ratio of 1+1 to a final concentration of 10mg/ml for alder and 25mg/ml for hazel. Control cells were incubated with DMEM diluted with physiological saline in the same ratio (1+1). The cell proliferation has been measured for three consecutive days at intervals of 20 minutes. Data are displayed as change of impedance ("Cell Index") over time, normalized to the point of time before sample addition.

Statistical analysis

Data were analysed using SPSS version 24 (SPSS Inc. Chicago, Ill., USA). For cell culture

experiments groups were built and compared using ANOVA. For post-hoc analysis Bonferroni correction was used. When the equal variances assumption has been violated Games-Howell post hoc tests were used instead. A significance level of $p=0.05$ was defined for all statistical analyses.

Results

Histamine content

The average histamine level of pollen amounted to 5.9 ng/ml for alder and 9.9 ng/ml for hazel. After irradiation with UV-light the histamine content raised substantially, the longer the irradiation period the higher the histamine content. The mean histamine level of alder pollen was 8.8 ng/ml after two hours, 9.6 ng/ml after four hours, 10.1 ng/ml after six hours for irradiation with UV-A light and 11.4 ng/ml after two hours, 13.1 ng/ml after four hours and 24.6 ng/ml after six hours for irradiation with UV-B light (see figure 1).

The mean histamine level of hazel pollen was 12.6 ng/ml after two hours, 16.6 ng/ml after four hours and 22.8 ng/ml after six hours of irradiation with UV-A light. For irradiation with UV-B light the levels were 18.1 ng/ml after two hours, 23.4 ng/ml after four hours and 39.5 ng/ml after six hours (see figure 2). The mean histamine level after 24 hours of irradiation with sunlight was 15.7 ng/ml for alder and 32.5 ng/ml for hazel pollen.

[Figure 1 near here]

[Figure 2 near here]

Cell culture

The Cell culture experiments revealed a decrease of cell viability of conjunctival cells after incubation with alder and hazel pollen.

Incubation with the non-irradiated alder pollen (10 mg/ml) did not lead to a significant decrease in cell viability ($p>0.05$, Fig 3) compared to the control.

When using pollen that have been preliminary irradiated for one day, the cell viability significantly decreased for UV-A and UV-B ($p<0.001$) compared to the control.

When comparing irradiated with non-irradiated pollen, the decrease in cell viability was also significant for UV-A ($p<0.001$) and UV-B ($p=0.039$).

Supernatants of pollen extracts were also tested: experiments revealed that they were less harmful than whole pollen suspensions. Compared to the control the decrease in cell viability with non-irradiated pollen supernatants was not significant ($p>0.05$).

When using pollen supernatants that have been preliminary irradiated for one day, the cell viability decreased significantly for UV-B ($p<0.001$) but not for UV-A ($p>0.05$) compared to the control.

When comparing pollen supernatants with and without irradiation, statistically significant results for UV-B ($p=0.003$) but not for UV-A could be observed ($p>0.05$; full descriptive results shown in table 1).

[Figure 3 near here]

Incubation with the non-irradiated hazel pollen (25 mg/ml) lead to a significant decrease in cell viability ($p=0.012$, Fig 4) compared to the control.

When using pollen that have been preliminary irradiated for one day, the cell viability significantly decreased for UV-A and UV-B ($p<0.001$) compared to the control.

When comparing irradiated with non-irradiated pollen, the decrease in cell viability was also significant for UV-A and UV-B ($p=0.001$).

As observed for alder pollen, the supernatants of hazel pollen extracts were also less harmful than whole pollen suspensions. Compared to the control the decrease in cell viability with non-irradiated pollen supernatants was not significant ($p=0.678$).

When using pollen supernatants that have been preliminary irradiated for one day, the cell viability decreased significantly for UV-A ($p=0.027$) and UV-B ($p<0.001$) compared to the control.

When comparing pollen supernatants with and without irradiation, statistically significant results for UV-B ($p=0.002$) but not for UV-A could be observed ($p=0.073$; full descriptive results shown in table 1).

[Figure 4 near here]

[Table 1 near here]

During the experiment all wells were evaluated repeatedly under the microscope, and two major things were apparent: (1) After incubation the pollen could not be fully removed despite several washing steps; (2) a marked loss of cells was detected for all the wells where

using irradiated pollen solutions, a minimal loss of cells when using non-irradiated pollen solutions and no loss of cells for the control at all (see figure 5).

[Figure 5 near here]

xCELLigence analysis

Proliferation assessment using the xCELLigence system revealed a strong influence of pollen on the ability of cells to grow and proliferate. In comparison to a normal proliferation rate of control cells, proliferation rate of cells incubated with pollen altered after contact with alder or hazel pollen solutions: while cells in culture media show a normal proliferation (see red lines in figures 6 and 7), cells treated with non-irradiated pollen stopped to proliferate after pollen contact which indicates that pollen have a cytostatic effect on conjunctival cells (see green lines in figures 6 and 7). Irradiated pollen exhibited a strong cytotoxic effect on conjunctival cells. Within a few minutes after test solution was added, cells detached from the surface of the wells, as indicated by the sharp decline of the growth curve. These results are consistent with the results of the MTS-tests. Effects were stronger for UV-A (blue lines) and UV-B light (pink lines), and sunlight effects were more or less the same as for non-irradiated pollen (turquoise lines).

[Figure 6 near here]

[Figure 7 near here]

Discussion

In our study we could demonstrate that UV-A light, UV-B light and sunlight are able to alter the composition of pollen. The analysis of the histamine content of alder and hazel pollen before and after irradiation revealed that histamine increases after irradiation. Histamine is a naturally produced component in living organisms such as plants, microbes and also pollen (20–23). In human bodies histamine is the major mediator of allergic reactions; high levels lead to itching, redness or burning of the eyes (24,25). Once pollen get in contact with the ocular surface it is known that they can stay there up to several hours (26). During this time every pollen grain will release histamine and other substances, which are able to destroy proteins of the tear fluid and thus may provoke different eye complaints (27). The number of patients that suffer from allergic eye complaints increases from year to year. We assume that UV light irradiated pollen might be a contributing co-factor for this increase; although, further tests are needed to confirm this theory. The experiments were carried out in spring when the irradiance of sunlight is lower, and also the sunshine duration is briefer than during the summer months. So it is assumed that the observed effects would be stronger for pollen species with flowering periods in summer or additionally also for other countries nearer the equator with constantly higher UV light levels.

Cell Culture

Allergic reactions typically cause symptoms in the lung, nose or eyes. The focus of this study lay on the effects considering the eyes, especially on the conjunctiva as this tissue is permanently exposed to the environment. MTS-tests revealed that incubation with pollen led to a decrease of cell viability and mitochondrial function of human conjunctival cells. The effects were stronger when using irradiated pollen compared to non-irradiated pollen. Additionally, incubation of conjunctival cells with pollen caused discontinuation of cell proliferation as assessed by the xCELLigence analysis system. It seems that the UV-light-

induced alterations of pollen ingredients make pollen more harmful for conjunctival cells. It is known that ingredients of pollen can cause several allergic and non-allergic reactions at the ocular surface. Pollen grains contain many proteolytic enzymes and oxidases, which produce reactive oxygen species (ROS) and lead to oxidative stress within minutes. Through pollen contact the release of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8) or tumor necrosis factor- α (TNF- α) and thus local immune responses in airway or conjunctiva can be triggered (28,29). It can be hypothesized that an irradiation of pollen may strengthen these effects and thus provoke more symptoms on eye, nose or lungs.

When using entire pollen suspensions with pollen grains, a loss of cells in the wells after the washing steps could be detected. The loss was not detectable for control cells, which indicates that pollen influence the adherence of cells. Pollen contain peptidases, which can disrupt epithelial tight junctions by degrading the extracellular domains of these proteins. This leads to an impairment of the epithelial barrier or cell membrane and thus to an influx of harmful substances into the cell and could be the reason for the detachment of the cells that has been observed (30). The fact that cells detach from the ground after contact with pollen has to be considered when interpreting the results: the MTS test measures the mitochondrial activity, which is directly correlated with the number of cells. If cells were washed away before the test there were less cells in the wells and thus a lower cell viability even if pollen had an effect or not. This is one of the limitations of this study; therefore, the results of the cell culture experiments should not be seen as definite values; however, it can be concluded that pollen have an effect on cell viability and that the effect is greater when pollen have been irradiated with UV-A or UV-B light before.

Conclusion

The experiments showed that UV light is capable of making pollen more harmful for conjunctival cells. It supports the theory that environmental factors play a contributing role in the development of allergic symptoms on the ocular surface.

It would be interesting to investigate whether these effects could also be observed for other pollen species. Further experiments will be needed to answer this question and to reveal possible other alterations of pollen ingredients due to UV light.

Geolocation information: Graz, Styria, Austria

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Figure captions

Fig 1. Histamine content of alder pollen after UV light and sunlight irradiation.

Fig 2. Histamine content of hazel pollen after UV light and sunlight irradiation.

Fig 3. Cell viability of CHANG cells + alder pollen (with and without UV-A and UV-B light irradiation for 1 day).

Fig 4. Cell viability of CHANG cells + hazel pollen (with and without UV-A and UV-B light irradiation for 1 day).

Fig 5. CHANG cells incubated with Pollen, photographed after two washing steps: A: control cells in DMEM; B: 10 mg/ml alder pollen, C: 10 mg/ml alder pollen that have been preliminary irradiated with UV-A light for 2 days; D: 10 mg/ml alder pollen that have been preliminary irradiated with UV-B light for 2 days; after washing steps a loss of cells was recognizable for irradiated pollen (pictures C and D).

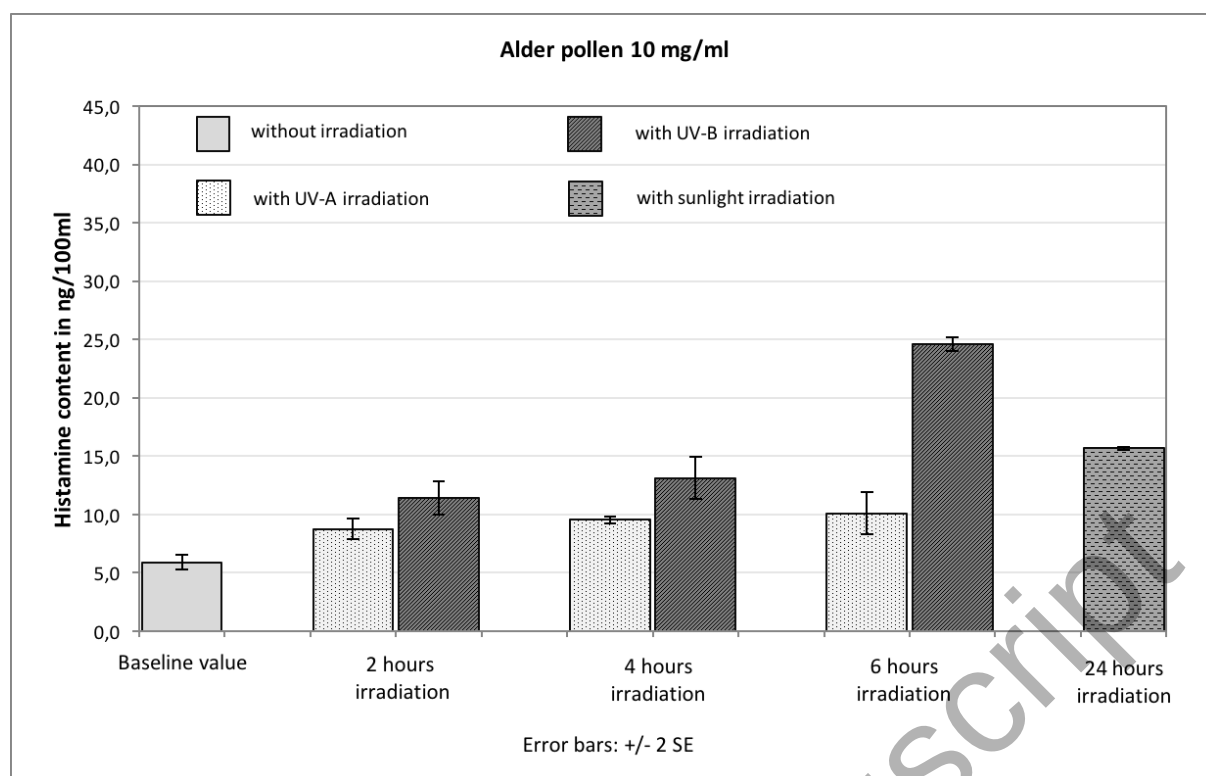
Fig 6. Cell proliferation after incubation with supernatant of irradiated and non-irradiated alder pollen. The treatment point indicates the time where solutions had been added to the cells.

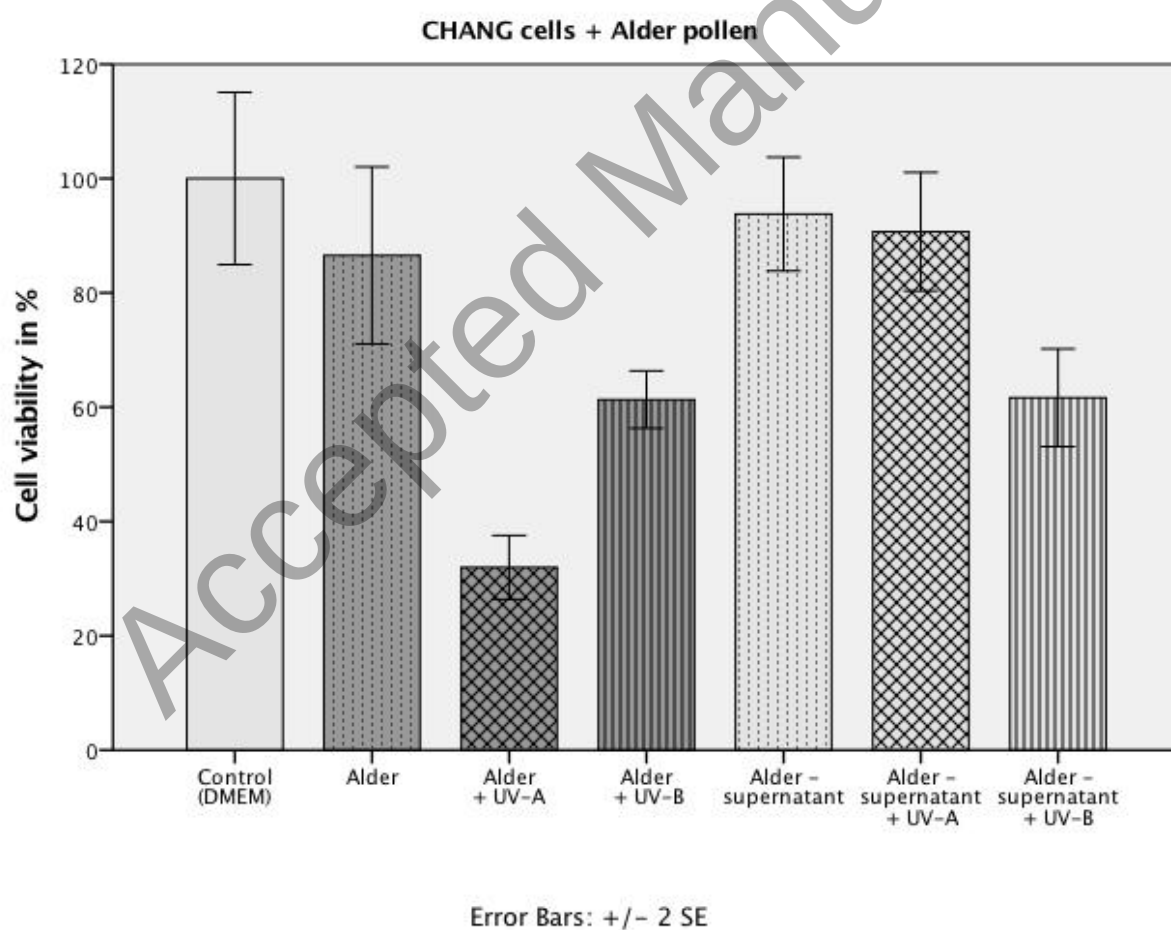
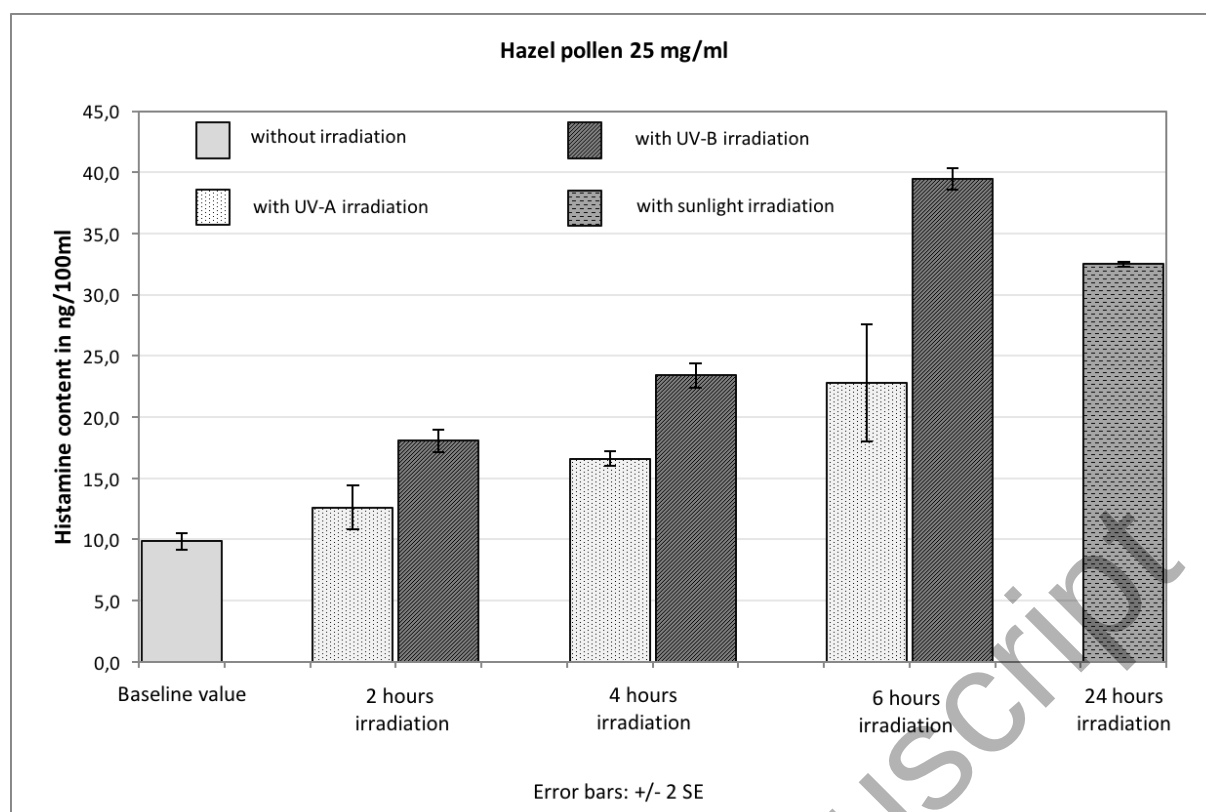
Fig 7. Cell proliferation after incubation with supernatant of irradiated and non-irradiated hazel pollen. The treatment point indicates the time where solutions had been added to the cells.

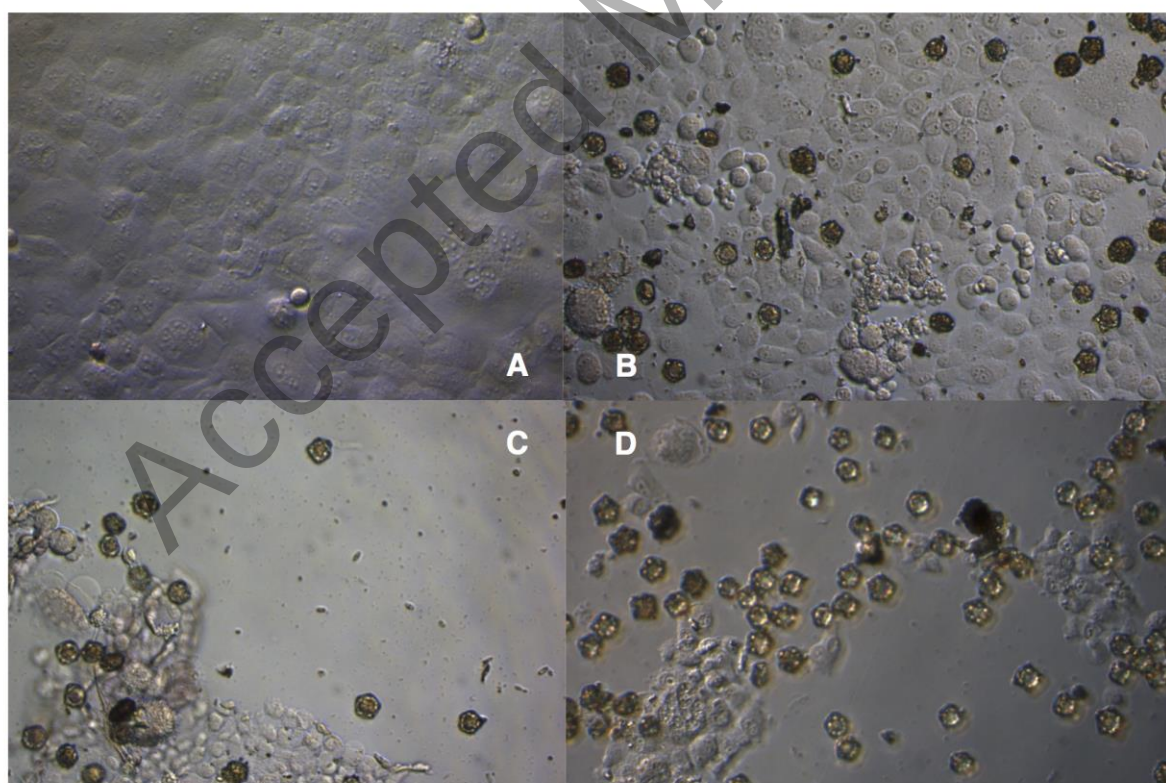
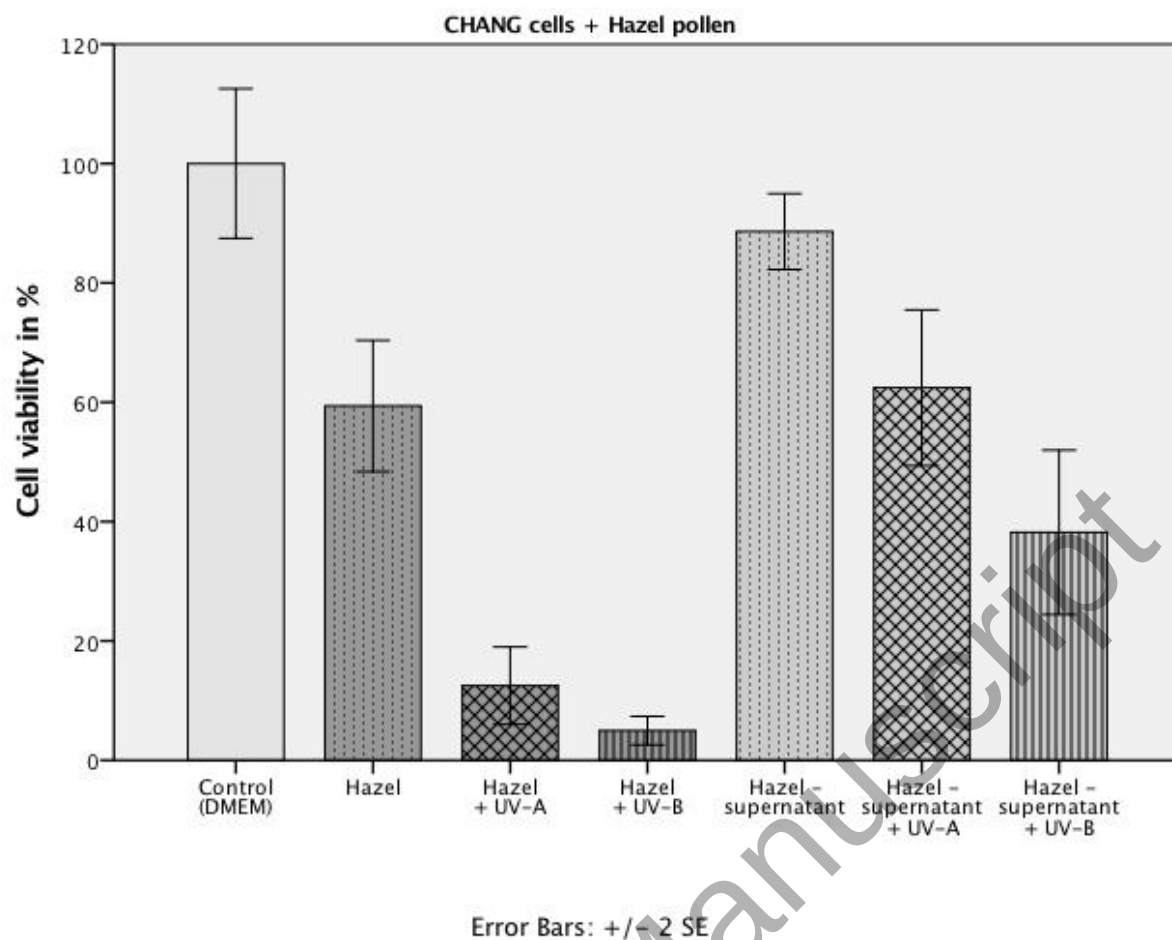
Table captions

Table 1: MTS results of CHANG cells incubated with irradiated and non-irradiated alder and hazel pollen.

Word count (including tables, references, figure captions): 4427 words







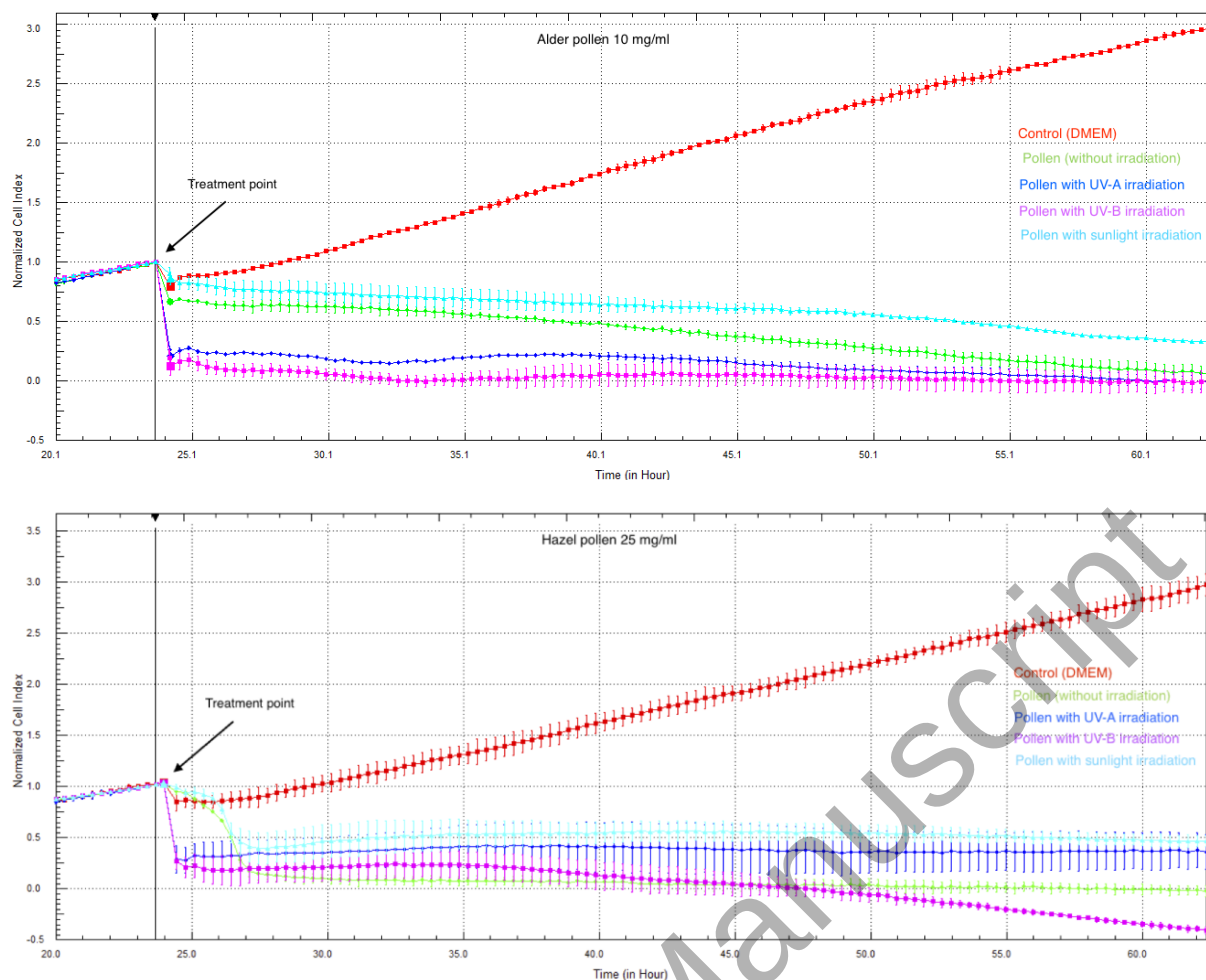


Table 2: MTS results of CHANG cells incubated with irradiated and non-irradiated alder and hazel pollen.

Alder	Cell viability in % (± 2 SE)	Hazel	Cell viability in % (± 2 SE)
CHANG (control)	100 (± 15.08)	CHANG (control)	100 (± 12.53)
Pollen suspension			
CHANG + Alder 10 mg/ml	86.55 (± 15.51)	CHANG + Hazel 25 mg/ml	59.37 (± 10.99) *
CHANG + Alder 10 mg/ml + UV-A	31.97 (± 5.58) * §	CHANG + Hazel 25 mg/ml + UV-A	12.50 (± 6.48) * §
CHANG + Alder 10 mg/ml + UV-B	61.33 (± 5.02) * §	CHANG + Hazel 25 mg/ml + UV-B	4.97 (± 2.38) * §
Pollen supernatant			
CHANG + Alder 10 mg/ml	93.79 (± 9.95)	CHANG + Hazel 25 mg/ml	88.59 (± 6.37)
CHANG + Alder 10 mg/ml + UV-A	90.70 (± 10.39)	CHANG + Hazel 25 mg/ml + UV-A	62.43 (± 13.04) *
CHANG + Alder 10 mg/ml + UV-B	61.64 (± 8.55) * §	CHANG + Hazel 25 mg/ml + UV-B	38.20 (± 13.76) * §

* Value indicates statistically significant difference ($p < 0.05$) between pollen and control

§ Value indicates statistically significant difference ($p < 0.05$) between non-irradiated pollen and irradiated pollen