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In Vitro Evaluation of the Biosafety of Hyaluronic Acid PEG Cross-Linked with Micromolecules of Calcium Hydroxyapatite in Low Concentration

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Keywords: hyaluronic acid; biosafety; calcium hydroxyapatite; human keratinocytes; Neauvia Stimulate

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Abstract

OBJECTIVE: Neauvia Stimulate is biocompatible, injectable hyaluronic acid (HA) filler (26 mg/ml) PEG cross-linked with 1% of calcium hydroxyapatite (CaHA) for facial soft-tissue augmentation that provides volume to tissues, followed by process of neocollagenesis for improving skin quality.

AIM: The aim of the present study is to evaluate the biosafety of the product (Lot. 160517-26-1/2 PEG) on human keratinocytes cultured in vitro.

MATERIAL AND METHODS: The experimental model proposed, despite being an in vitro system, allows the derivation of useful information to predict the possible activity of the product in further in vivo application. Human keratinocytes (HaCaT cells) were treated with the product for 24h at increasing concentrations of product respect to control (untreated cells).

RESULTS: The biosafety of the product to be tested has been evaluated performing different methods: MTT test, NRU test, Kenacid Blue assay. Moreover, any possible effect on the structure, morphology, and viability of cells has been evaluated.

CONCLUSION: In conclusion, the results obtained by the different methods show that the product Neauvia Stimulate® does not cause any cytotoxic effect and does not affect the correct structure and morphology of cells cultures.

Introduction

Neauvia Stimulate (MatexLab SA, Lugano, CH) is a product which combines pure hyaluronic acid of probiotic origin (*Bacillus Subtilis*) cross-linked with PEG (poly-ethylene-glycol) and micromolecules (10-12 μm size) of calcium hydroxyapatite in low concentration (1%). The product could be considered a "composite" filler (completely biocompatible and degradable) with both volumizing effects, typical of the HA filler cross-linked polymer [1][2][3], and a collagenesis activity. The latter is obtained by the action of calcium hydroxyapatite that stimulates the

skin self-production of collagen [4][5][6].

The aim of the present work is to evaluate the *in vitro* biosafety, in term of cytotoxicity and modification of the cellular structure and morphology, after treating human keratinocytes cultured *in vitro* with the product Hyaluronic Acid Hydrogel 26 mg/ml PEG cross-linked with Calcium Hydroxyapatite 1% (Lot. 160517-26-1/2 PEG), named Neauvia Stimulate. The experimental model proposed, despite being an *in vitro* system, allows the derivation of useful information to predict the possible activity of the product in further *in vivo* applications.

Materials and Methods

Sample preparation

The product Neauvia Stimulate was weighed and dissolved at the concentration of 5 mg/ml in complete medium constituted by DMEM with 10% fetal bovine serum (FBS), one mM L-glutamine and antibiotics (100 UI/ml penicillin and 100 µg/ml streptomycin). SLS (Sodium Lauryl Sulphate), well-known cytotoxic substance, was used as positive control and was prepared as described for the product.

Cell cultures

Keratinocytes are the most represented cell type in the epidermis cells. They grow from the base of the epidermis where cells multiply and then migrate to the surface of the skin producing lipids, natural factors of hydration and keratin. Human immortalised keratinocytes used in the assay were a human cell line (HaCaT, code BS CL 168). The cell line was grown in conditions of complete sterility and maintained in incubation at 37°C with 5% carbon dioxide (CO_2) atmosphere.

Cytotoxicity assay (MTT test)

The MTT test is a colourimetric cytotoxicity assay used to test cell proliferation and viability based on mitochondrial efficiency. The MTT, a tetrazolium salt that, in case of cells metabolical activity, is reduced from the highly reducing mitochondrial environment of viable cells by the action of mitochondrial dehydrogenase. MTT reduction leads to the formation of formazan crystals (Fig. 1) - insoluble in the culture medium, but soluble in DMSO - which gives the typical purple colour to the mitochondria of viable cells. Contrarily, in suffering or dead cells, since active mitochondria are lacking, MTT will not be reduced resulting in a less intense purple colour [7]. For the direct relationship between cellular respiration and viability, MTT is considered a good assay to identify the non-cytotoxic concentrations of the product Neauvia Stimulate.

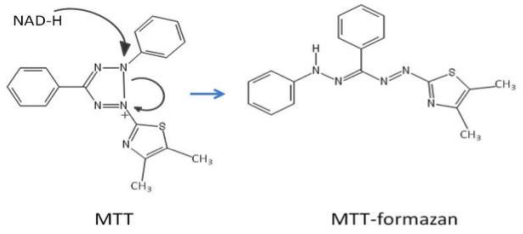

Figure 1: MTT reduction in formazan. The reaction is catalysed by succinate dehydrogenase

For the preparation of the assay, HaCaT cells were homogeneously seeded in 96-well plates at a density of 1.5 x 10^4 cells-per-well and incubated at 37°C with 5% CO_2 humidified atmosphere. After 24 h, cells were treated (six replicates for each of the eight different concentrations) starting with a concentration of 5 mg/ml up to the final one of 0.039 mg/ml through a serial dilution of 1:2. Cells treated with SLS were used as positive control (Ctrl+, starting concentration 5 mg/ml in complete medium).

Incubation was performed for 24 h. Following, ten µl of MTT stock (5 mg/ml in PBS) were added to HaCaT cells at 37°C for two h. The medium was then removed, and 100 µl of DMSO was added to the cells. Subsequently, absorbance was measured at a wavelength of 570 nm using a microplate reader. Cell viability was calculated measuring the difference in optical density of each of the eight concentrations of the tested product concerning control (untreated cells) (8). Data were processed using Photox v. 2.0 for IC_{50} calculation, which is the concentration of the product that determines the 50% of cell viability.

Cytotoxicity assay (NRU test)

The NRU cytotoxicity assay is a colourimetric test based on the ability of viable cells to incorporate the dye in lysosomes [9]. Neutral Red (NR, Sigma) is a weak cationic dye that readily penetrates cell membranes and accumulates intracellularly in lysosomes, thereby providing direct information on the cell membrane integrity and, indirectly, on the viability of cells. For the preparation of the assay, cells were seeded and treated as previously described. At the end of the treatment, cells were examined under a phase-contrast microscope and washed in PBS. One hundred µl of the NR medium was then added and cells were incubated for three h at 37°C, 5% CO_2. After the medium has been discarded, an acetic acid solution was added to extract the NR from cells, and the reading of the absorbance was performed at 540 nm wavelength using a microplate reader (Tecan Sunrise). Cell viability was calculated as previously described.

Cytotoxicity assay (Kenacid Blue assay)

The Kenacid Blue test is a colourimetric cytotoxicity assay used to test cell viability based on the ability of a dye to bind cellular proteins [10]. The Kenacid Blue assay system measures total biomass by staining proteins (total biomass) with a specially developed dye creating a simple, accurate, and highly reproducible test. For the preparation of the assay, cells were seeded and treated as previously described. Cells were then washed with PBS and fixed with a 3% glutaraldehyde solution for 20 min at room temperature. Fifty µl of the Kenacid Blue Acid Stain Solution was added to each well for 20 min at room temperature. At the end of the incubation, the medium was then discarded, and cells were washed with an acetic acid solution (5% in 10% ethanol). Finally, 100 µl of Kenacid Blue Assay Extraction Solution was added to each well for 20 min at room temperature with gentle shaking. Subsequently, the absorbance was read at 570 and 690 nm wavelength using a microplate reader. Cell survival was calculated as previously described.

Evaluation of cell viability

Cell viability has been evaluated by "LIVE/DEAD" (Life Technologies Ltd) commercial kit that uses two different fluorescent probes, the red-fluorescent nucleic acid stain, propidium iodide, and the SYTO® nine green-fluorescent nucleic acid stain for the determination of live and dead cells. The SYTO 9 penetrates all cells, those with intact and damaged membranes. In contrast, propidium iodide penetrates only cells with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. For the preparation of the assay, cells were homogeneously seeded onto glass coverslips (22 x 22 mm), placed inside Petri dishes (35 x 10 mm), at a density of 1 x 10^5 cells, and incubated at 37°C, with 5% CO_2 humidified atmosphere. After 24 h, cells were treated with two concentrations of the product, equal to 2.5 mg/ml and 1.25 mg/ml, demonstrated to be non-cytotoxic and with the best solubility in the culture medium. After 24 h treatment, an appropriate mixture of the two dyes was added to each dish for 15 min. Samples were then stored protected from light and analysed using fluorescence microscopy.

Evaluation of cell morphology

The hematoxylin and eosin (EE) staining, commonly used in the microscopic study of animal tissues and histopathology routine, has been used to evaluate any possible effect on cell cultures after the treatment with the product Neauvia Stimulate. For the preparation of the assay, cells were seeded and treated as described in the previous paragraph. At the end of the treatment, cells were washed and fixed in methanol; subsequently, a 1% hematoxylin-eosin solution has been added to each slide. After having carefully removed the stain, coverslips has been mounted on microscope slides to promote the drying.

Evaluation of cell structure

For the study on the effects on the cytoskeleton due to the treatment with the product Neauvia Stimulate®, a fluorescent Phalloidin molecule has been used to detect the microfilaments of F-actin [11] in immunofluorescence. For the preparation of the assay, cells were seeded and treated as previously described. After the treatment, HaCaT cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then incubated with the antibody Alexa Fluor® 488 phalloidin at room temperature. After this period, the excess of phalloidin and any residues were removed through two further washes in PBS and the nuclei stained with Hoechst 33258. The coverslips were then mounted on slides and analysed by confocal microscopy.

Results

Results are reported in charts, and images containing the measurements obtained by cell cytotoxicity assays and the evaluation of cell viability, structure and morphology after treatment with the product Neauvia Stimulate concerning control HaCaT cells. Data represented as the mean of at least two independent experiments performed in single.

Evaluation of cell cytotoxicity

HaCaT cells were incubated and treated for 24 h with eight different concentrations of the product Neauvia Stimulate®, along with an appropriate positive control, to identify a possible cytotoxic effect on cell cultures and the concentrations to use in the following assay.

Fig. 2 shows the results of the cytotoxicity tests. It is possible to note that, at all concentrations tested and in all the three assays, the product Neauvia Stimulate did not cause a decrease in cell viability so to calculate the IC_{50} value and, consequently, the product did not show cytotoxic activity.

The concentrations of 1.25 mg/ml and 2.5 mg/ml were chosen for the following assays for their best solubility in the culture medium.

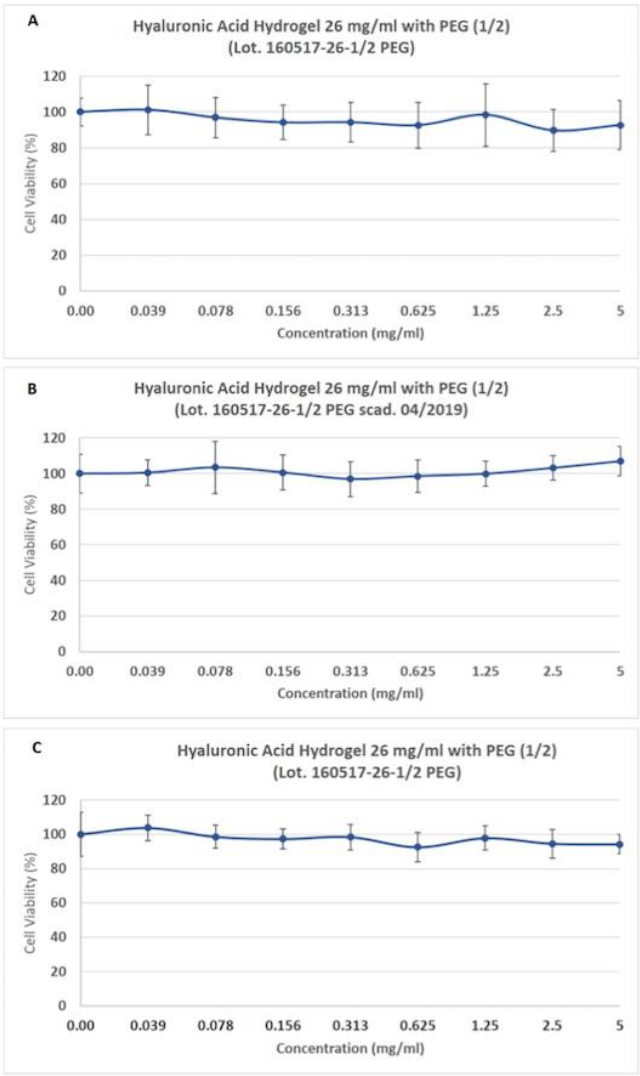

Figure 2: Graphics of cell viability obtained after 24 h treatment of HaCaT cells with the product Neauvia Stimulate®. (A): MTT test; (B) NRU test; (C) Kenacid Blue assay

Evaluation of cell viability

HaCaT cells were incubated and treated for 24 h with the concentrations of 1.25 mg/ml and 2.5 mg/ml of the product Neauvia Stimulate®, along with an appropriate positive control. Fig. 3 shows the representative images obtained with a fluorescence microscope of the results of the LIVE/DEAD assay. Analysing the images, it is clear that none of the two concentrations tested determines a variation in cell viability and that the number of stained cells in green (live) is comparable to the control (untreated cells). In contrast, it is evident an alteration of viability after treatment with SLS, with a decrease in the total number of observed cells and increased mortality (cells stained in red) compared to the control.

Evaluation of cell morphology

HaCaT cells were incubated and treated for 24 h with the two previously described concentrations of the product Neauvia Stimulate®, along with an appropriate positive control.

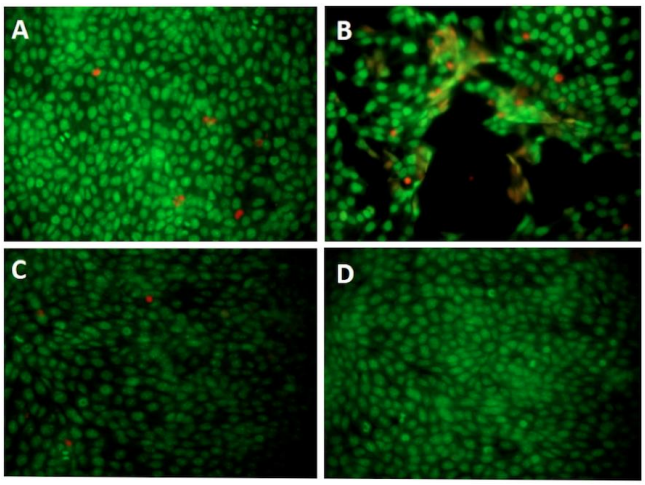

Figure 3: Images obtained using a fluorescence microscope of the staining with LIVE/DEAD kit after 24 h treatment with the product Neauvia Stimulate®. A) Ctrl (untreated cells); B) SLS (Ctrl+); C) product 2.5 mg/ml; D) product 1.25 mg/ml

Fig. 4 shows the representative images in optical microscopy of the results of the Hematoxylin-Eosin test for each tested condition. None of the two concentrations of the product does alter the cell morphology when compared with the untreated control. It is also evident an alteration of the cell morphology induced by the SLS, with a remarkable decrease in the total number of observed cells and a modification of the normal morphology of the HaCaT cells compared to the negative control.

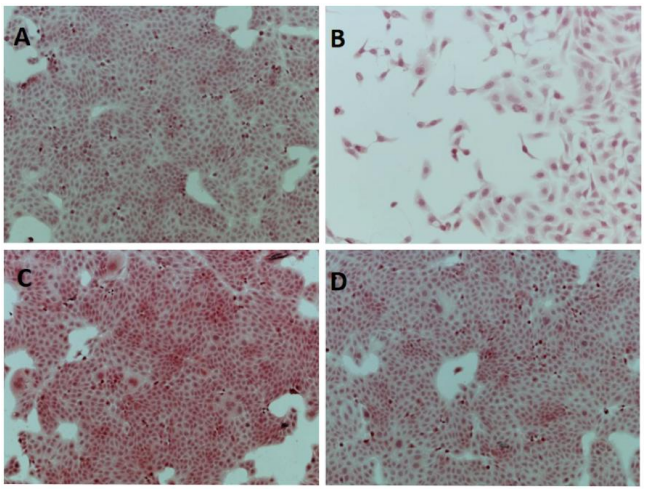

Figure 4: Optical microscopy images related to EE staining after 24 h treatment of HaCaT cells with the product Neauvia Stimulate®. A) control (untreated cells); B) SLS (Ctrl +); C) product 2.5 mg/ml; D) product 1.25 mg/ml

Evaluation of cell structure

HaCaT cells were incubated and treated for 24 h with the two previously described concentrations of the product Neauvia Stimulate®, along with an

appropriate positive control. Fig. 5 shows the representative images in confocal microscopy of the results obtained by the cytoskeleton's structure evaluation. Analysing the images, it is clear that none of the two tested concentrations determines an alteration in the structure of cytoskeleton (stained in green) compared to the control (untreated cells). In contrast, it is evident an alteration of the structure after treatment with SLS, with a decrease of the F-actin labelled and a decrease in the total number of observed cells.

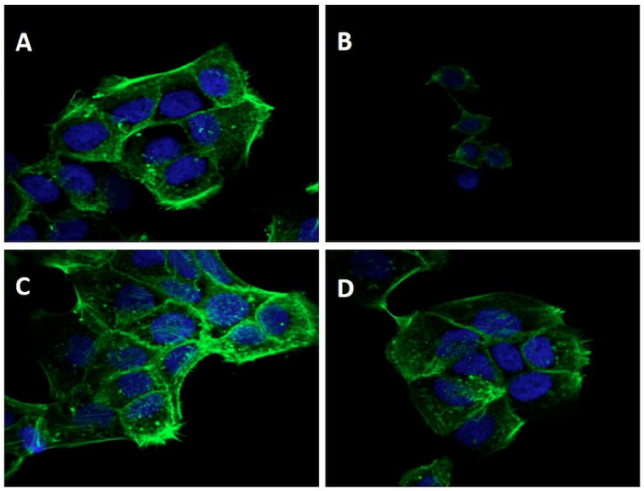

Figure 5: Images obtained using a confocal microscope of the staining with Phalloidin after 24 h treatment with the product Neauvia Stimulate®. A) Ctrl (untreated cells); B) SLS 0.1 mg/ml (Ctrl+); C) product 2.5 mg/ml; D) product 1.25 mg/ml

Discussion

From the results obtained using *in vitro* tests, we can conclude that the product Neauvia Stimulate® does not induce any cytotoxicity effect after 24 h treatment in human keratinocytes at the tested conditions. Moreover, the analyses performed with the two concentrations resulted to be with the best solubility in cell culture medium, do not cause any alteration of cell viability, cell morphology, and structure, showing results comparable to control cells, which were not treated.

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