IN VITRO EVALUATION OF COLLAGEN PRODUCTION ON HUMAN FIBROBLASTS TREATED WITH HYALURONIC ACID PEG CROSS-LINKED WITH MICROMOLECULES OF CALCIUM HYDROXYAPATITE IN LOW CONCENTRATION

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Neauvia Stimulate® is a 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 a process of neocollagenesis for improving skin quality. The aim of the present study is to evaluate the possible modulation of collagen synthesis after treating human fibroblasts cultured *in vitro* with the product (Lot. 160517-26-1/2 PEG). 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 fibroblasts (PEU cells) were treated with the product for 24 h at increasing concentrations of compared to control (untreated cells). The modulation of collagen synthesis was evaluated using a specific colorimetric kit (Sircol, Soluble Collagen Assay Kit). Increment of collagen production, 37.62% and 97.39% at concentrations of 1.25 mg/ml and 2.5 mg/ml of product, respectively, was considered to be statistically significant (*p values ≤0.05 and **p values ≤0.01) when compared with control (untreated cells). In conclusion, Hyaluronic Acid Hydrogel 26 mg/ml PEG cross-linked with calcium hydroxyapatite in low concentrations (1%) determines a statistical increment in neocollagenesis.

Neauvia Stimulate® (MatexLab SA, Lugano, CH) is a product which combines pure hyaluronic acid of probiotic origin (Bacillus Subtilis) cross-linked with PEG (poly-ethylen glycol) and micro molecules (10-12 μm) of calcium hydroxyapatite in low concentration (1%). The product could be considered a "hybrid" filler (completely biocompatible and biodegradable) with both a volumizing effect, 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, 9).

The aim of the present work is to evaluate the possible modulation of collagen synthesis after treating human fibroblasts cultured *in vitro* with the Hyaluronic Acid Hydrogel 26 mg/ml PEG cross-linked with Calcium Hydroxiapatite 1% (Lot. 160517-26-1/2 PEG) product. 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.

Key words: hyaluronic acid, calcium hydroxyapatite, neocollagenesis, human fibroblasts, collagen synthesis, Neauvia Stimulate®

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MATERIALS AND METHODS

Sample preparation

Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) was weighed and dissolved at the concentration of 5 mg/ml in complete medium constituted by MEM with 10% fetal bovine serum (FBS), 1 mM L-glutamine and antibiotics (100 UI/ml penicillin and 100 µg/ml streptomycin). SLS (Sodium Lauryl Sulphate), a well-known cytotoxic substance was used as positive control and was prepared as described for the product.

Cell cultures

Fibroblasts are cells of the connective tissue, able to secrete extracellular matrix components. Normal embryonic fibroblasts used in the assay were a human cell line (PEU, code BS CL 97). 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)

MTT test is a colorimetric cytotoxicity assay used to test cell proliferation and viability based on mitochondrial efficiency. MTT is a tetrazolium salt that, in case of metabolic cell activity, it reduces from the mitochondrial environment of viable cells by mitochondrial dehydrogenase. MTT reduction leads to the formation of formazan crystals (Fig. 1), insoluble in culture medium but soluble in DMSO, which gives the typical purple colour to the mitochondria of viable cells. Contrarily, since active mitochondria is lacking in suffering or dead cells, MTT will not reduce, 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 Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) product.

For the preparation of the assay, PEU 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 in a 5% CO_2 humidified atmosphere. After 24 h, cells were treated (6 replicates for each of the 8 different concentrations) starting with a concentration of 5 mg/ml up to the final one of 0.039 mg/ml trough 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, 10 µl of MTT stock (5 mg/ml in PBS) were added to PEU cells at 37°C for 2 h. The medium was then removed, and 100 µl of DMSO were added to the cells. Subsequently, absorbance was measured at a wavelength of 570 nm using a microplate reader (Tecan Sunrise). Cell viability was calculated measuring the difference in optical density of each of the eight concentrations of the tested product with respect to control (untreated cells) (8).

Evaluation of collagen synthesis

The capability of Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) to modulate the collagen production, was evaluated in human fibroblasts (PEU cells) after 24 h treatment, using a colorimetric kit (Sircol, Soluble Collagen Assay Kit). For the preparation of the assay, cells were homogeneously seeded in 24-well plates at a density of 8x10^4 cells-per-well and incubated at 37°C in a 5% CO_2 humidified atmosphere.

After 48 h, two of the tested product concentrations, 1.25 and 2.5 mg/ml, were chosen showing to be non-cytotoxic and best soluble (MTT test). Untreated cells (CTRL) were used as control. At the end of incubation, 200 µl of Tris-HCl, pH 7.4, containing polyethylene glycol, were added to the recovered supernatant for the isolation and concentration of collagen, then stored overnight at 0 to 4°C. Further, samples were centrifuged at 12,000 rpm and incubated for 10 minutes with Sircol dye reagent able to bind collagen. During this time, a collagen-dye complex was formed and precipitated out from the soluble unbound dye. Finally, after a step of centrifugation (12.000 rpm for 10 min) a reagent containing sodium hydroxide 0.5 M capable of solubilizing the precipitate collagen was added; 200 µl of sample were then transferred into a 96-well plate for spectrophotometric optical density reading at a wavelength of 555 nm.

RESULTS

Results are reported in tables and charts containing the measurements of collagen synthesis after treatment with the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) with respect to control in PEU cells. Data are presented as mean ± standard deviation (SD) of at least 2 independent experiments performed in single.

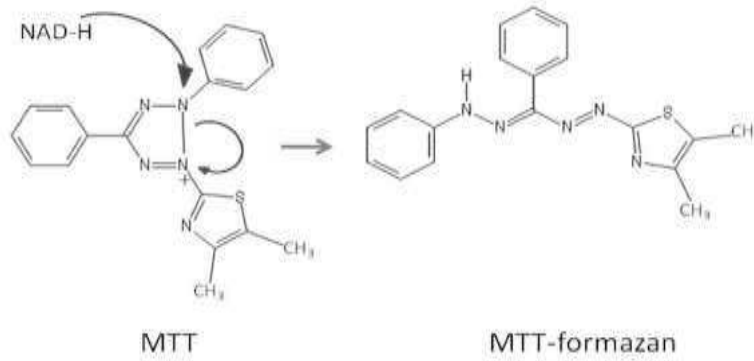

Fig. 1. *MTT reduction in formazano. The reaction is catalyzed by succinate dehydrogenase.*

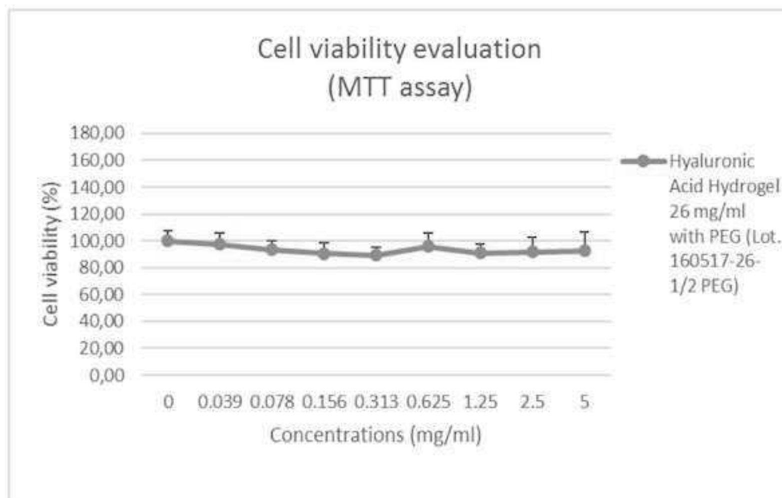

Fig. 2. *Graphic of cell viability obtained after 24 h treatment of PEU cells with the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG).*

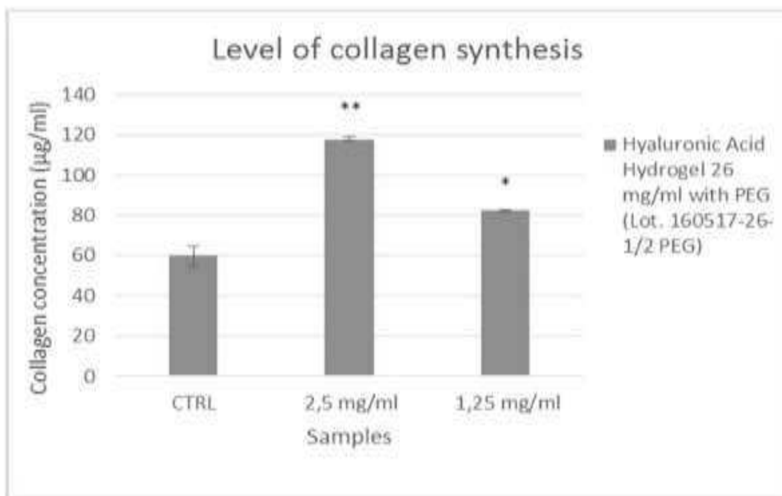

Fig. 2. *Graphic of cell viability obtained after 24 h treatment of PEU cells with the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG).*

Evaluation of cell viability

PEU cells were incubated and treated for 24 h with 8 different concentrations of the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG), along with an appropriate positive control, in order to identify the concentrations to use in the following assay. Fig. 2 shows the results of the cytotoxicity test. It is possible to note that, at all concentrations tested, the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517- 26-1/2 PEG) did not show cytotoxic activity.

Table I shows the measured data expressed as a percentage compared to control (untreated cells) for each product concentration tested. It is possible to note that, at all concentrations tested, the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) did not show cytotoxic activity; the concentrations of 1.25 mg/ml and 2.5 mg/ml were chosen for the following assay for their best solubility in the culture medium.

Evaluation of collagen synthesis

PEU cells were treated (24 h incubation) with two different concentrations of the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) (2.5 mg/ml and 1.25 mg/ml) in order to study a possible modulation of collagen levels. Fig. 3 shows the treatment of PEU cells with the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) at concentrations of 1.25 mg/ml and 2.5 mg/ml. The results obtained demonstrate that both concentrations determine an increase in the level of synthesized collagen equal to 37.62% and 97.39%, respectively, after 24 h treatment of PEU cells, compared with control (CTRL, untreated cells).

CONCLUSIONS

From the results obtained using *in vitro* tests, we can conclude that the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG), induced a statistically significant increase in the collagen production after treatment with the two concentrations tested after 24 h treatment in human fibroblasts. In particular, the product Hyaluronic Acid Hydrogel 26 mg/ml with PEG (Lot. 160517-26-1/2 PEG) determines a statistically increase in collagen production of 37.62% and 97.39% after treatment with 1.25 mg/ml and 2.5 mg/ml of product, respectively.

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