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Research Article

Characterization and *In Vitro* Evaluation of Injectable Collagen Biostimulators

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ABSTRACT

Dermal fillers represent a growing field in regenerative medicine, offering promising solutions for tissue regeneration and aesthetic enhancement. The objective was to assess cell viability and the biosynthesis of collagen in fibroblasts treated with biostimulators and characterize the morphology and zeta potential of the dermal fillers. Cells were exposed to the biostimulators Ellansé®, Radiesse®, Rennova® Diamond, Rennova® Elleva and Sculptra® in concentration of 0.5, 1.0 or 1.5 mg/ml of the active ingredient. The colorimetric cell viability test using the tetrazolium salt method (MTT assay) and the quantification of collagen synthesis after Picrosirius Red staining were performed after two and ten days of treatment, respectively. The characterization of the biomaterials was performed by optical microscopic analysis and the determination of the zeta potential. The results indicated no improvement in fibroblast MRC-5 viability after 2 days of contact with biostimulators. However, a significant increase in collagen synthesis was observed in fibroblasts treated with the biostimulators Radiesse® and Sculptra® at all concentrations and Rennova® Elleva (at 1.0 and 1.5 mg/ml) in comparison to the control. Hydroxyapatite particles (Radiesse® and Rennova® Diamond) had regular spherical shapes similar to polycaprolactone (Ellansé®), whereas poly-L-lactic acid (PLLA) particles (Rennova® Elleva and Sculptra®) had an irregular shape and bigger size. Rennova® Elleva and Sculptra® demonstrated highly stable zeta potential, while Ellansé® and Radiesse® exhibited relative stability and Rennova® Diamond exhibited instability. In conclusion, this study contributes with regard to the action of biostimulators without inflammatory cells. No product or dose of the fillers exhibited cytotoxicity. A difference was found in the biosynthesis of collagen between hydroxyapatite brands (Radiesse® and Rennova® Diamond) and no differences with PLLA brands (Rennova® Elleva and Sculptra®) when compared to each other. The characterization results revealed that the particles presented micrometric size with negative net charge and variable stability against coalescence.

Keywords: Aging; Biomaterials; Cell culture; Cell viability; Collagen; Dermal fillers; Fibroblasts; Skin

1. Introduction

Skin health is essential for overall well-being, serving as the body's primary barrier against environmental stressors, pathogens and physical injury. However, aging, environmental exposure and lifestyle factors can compromise skin integrity, leading to reduced elasticity, moisture loss and impaired wound healing. These challenges highlight the need for innovative approaches in regenerative medicine, biomaterials and dermatological treatments to maintain skin function, enhance repair mechanisms and improve quality of life[1].

The science of tissue regeneration has advanced significantly with the development of bioactive biomaterials offering promising strategies to counteract the effects of skin aging, a complex process influenced by intrinsic factors, such as genetics and cellular senescence and extrinsic factors, including UV radiation and pollution[2]. By promoting cellular renewal, enhancing collagen synthesis and modulating inflammatory responses, bioactive biomaterials have the potential to mitigate structural and functional deterioration associated with aging skin such as injectable biostimulants[3]. These substances, when interacting with tissue cells, induce repair and regeneration processes, offering new perspectives for the treatment of various conditions, from skin aging to the repair of damaged tissues. [4] This set of treatments harmonizes the smile and balances the face, leading to an improvement in self-esteem[5]. Among facial fillers, biostimulators have attracted considerable attention, the aim of which is to reestablish lost volume and actively affect deeper layers of the skin by stimulating the formation of new collagen[6-8].

Biodegradable biostimulators are absorbed through naturel phagocytic mechanisms and can last between 18 months and five years. This category includes poly-L-lactic acid (PLLA), calcium hydroxyapatite (CaHA) and polycaprolactone (PCL)[9,10].

PLLA is sold as Sculptra® Galderma (Switzerland) or Rennova® Elleva (Croma Pharma GMBH, Austria) and is an injectable polymer formed by microparticles that stimulate the biosynthesis of collagen. PLLA is produced from the fermentation of sugar from corn and its particles measure around 40-63 µm in diameter. The composition of these products includes sodium carboxymethylcellulose and non-pyrogenic mannitol[11,12].

Calcium hydroxyapatite is used as a synthetic injectable collagen biostimulator, known in Brazil by the brand names Radiesse® (BioForm Medical, USA) and Rennova® Diamond (Croma Pharma GMBH, Austria). These biostimulators are composed of 30% synthetic microspheres of calcium hydroxyapatite, which are spherical and uniform, ranging in diameter from 25 to 45 µm and 70% an aqueous vehicle composed of sodium carboxymethylcellulose, sterilized water and glycerin[8,13,14].

Ellansé® (Sinclair, The Netherlands) is composed of 30% synthetic PCL microspheres and 70% aqueous carboxymethylcellulose vehicle. The microspheres have a diameter of 25-50 µm, are smooth and spherical, with a uniform size, unlike PLLA particles, which have non-uniform, rough, flat morphology and sharp format[15-17].

The implementation of collagen-inducing biomaterials has enabled the correction of facial depressions and imperfections, achieving gradual three-dimensional orofacial harmonization with natural results, leading to an improvement in self-esteem[18].

Wellbeing and improved self-esteem are of the utmost importance to the population. Thus, when well indicated and executed, esthetic procedures contribute significantly to the improvement of the health of the population by managing signs of aging[5-7]. Discontentment with one's self-image has harmful biopsychosocial effects that can exert an influence on behavior and how one expresses oneself. Studies have demonstrated that individuals with a poor self-image can suffer from an inferiority complex, non-acceptance and impotence[5,6].

The search for a functional and esthetically pleasing facial image has made biostimulators a precious technology in orofacial harmonization, playing an important role in society[19,20]. These biomaterials have been increasingly used to correct structural defects of the face and improve the appearance of patients. Due to the broad gamut of available products, there is a need for studies that explore and compare the mechanisms of these biomaterials.

Although biostimulators have positive effects in the induction of collagen[8-10,12-17], no studies have compared different brands with regards to fibroblast viability and the synthesis of collagen. Therefore, the aim of the present study was to assess cell viability and the biosynthesis of collagen in fibroblasts treated with biostimulators and describe the morphology and zeta potential of the main commercial biostimulators.

2. Materials and Methods

An in vitro laboratory experimental study was conducted. The biostimulators analyzed were Rennova® Elleva (Croma Pharma GMB, Austria), Rennova® Diamond (Croma Pharma GMBH, Austria), Ellansé® (Sinclair, The Netherlands), Sculptra® (Galderma, Switzerland) and Radiesse® (BioForm Medical, USA). The biostimulators were prepared following the recommendations of the manufacturers. Rennova® Elleva and Sculptra® were dispersed in injection water and Radiesse®, Rennova® Diamond were Ellansé® dispersed in saline solution.

2.1. Cell culture

MRC-5 fibroblasts (human fetal lung) were maintained in the culture media low- glucose Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 2.5 g/l of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich, St Louis, Missouri, USA), 3.7 g/l of sodium bicarbonate (Neon Comercial, Suzano, SP, Brazil), 10% heat-inactivated fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (Sigma-Aldrich, St Louis, Missouri, USA), pH 7.4. The cells were cultivated in plastic culture flasks (Kasvi Importação e Distribuição de Produtos Para Laboratórios Ltda., São José dos Pinhais, Brazil) and incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was changed two to three times per week.

Once the cells achieved confluence, trypsinization was carried out using a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (obtained from Sigma- Aldrich, St. Louis, Missouri, USA) at a concentration of 0.25% in phosphate-buffered saline (PBS) for a duration of three to five minutes. PBS was produced with 8 g/l sodium chloride (NaCl) (Neon Comercial, Suzano, SP, Brazil), 0.2 g/l potassium chloride (KCl) (Cromato Produtos Químicos, São Paulo, Brazil), 0.2 g/l anhydrous dibasic sodium phosphate (Na_2HPO_4) (Dinâmica, São Paulo, Brazil) and 1.1

g/l anhydrous monobasic sodium phosphate (NaH_2PO_4) (Synth, São Paulo, Brazil). This procedure was performed to possibility the cell counts in a Neubauer chamber[21].

2.2. Biostimulators cells treatment

The MRC-5 cells were seeded at a density of 7,500 cells per well for the viability test and 2,000 cells per well were sown for the collagen assessment in 96-well culture plates (Kasvi, Brazil). The biostimulators were suspended in 200 µl of culture media.

After preparation, the viability and collagen biosynthesis tests were performed with concentrations of 0.5, 1.0 and 1.5 mg/ml of the active ingredient suspended in 88.5% of cell culture medium and 11.5% saline solution or injection water (as recommended by the manufacturer). The control for Rennova® Elleva and Sculptra® was 11.5% injection water in the culture medium. The control for Radiesse®, Rennova® Diamond and Ellansé® was 11.5% saline solution.

2.3. Cell viability test

Cell viability was determined using the tetrazolium salt method (MTT assay) (Sigma-Aldrich, China), which is a colorimetric test involving 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide. After a period of two days, the supernatant was removed and 75 µl/well of MTT at a concentration of 0.25 mg/ml were added to 96-well culture plates. After three hours of incubation, the reagent was removed and 200 µl/well of dimethyl sulfoxide (DMSO, Synth, Diadema, Brazil) were added to dissolve the crystals that had formed, followed by agitation for ١٠ minutes. Absorbance was then read with the aid of a spectrophotometer (Multiskan Thermo Scientific, Shangai, China) at wavelengths of 570 nm and 630 nm. The result was calculated by the difference between wavelengths. Cell viability was expressed as mean absorbance ± standard deviation (SD)[21,22].

2.4. Biosynthesis of collagen

The quantification of the biosynthesis of collagen was performed based on a chromogenic precipitation reaction between collagen and Picrosirius Red (PR) stain (0.1% Direct Red 80 [Sigma-Aldrich, St Louis, Missouri, USA] in 1.3% picric acid), followed by the spectrophotometric reading of the biosynthesis of the protein. PR is a dye that specifically binds to the helicoidal structure of fibrillar collagen[23-25].

Treatment of the cells with the biostimulators was performed in 96-well culture plates with 80 to 90% confluence. The cells were treated for 10 days, with the medium containing the biomaterials exchanged every three days. After 10 days of the culturing of MRC-5 fibroblasts, the medium was removed and the wells were washed twice with 300 µl of PBS. Seventy-five µL of 4% paraformaldehyde (Sigma-Aldrich, St Louis, Missouri, USA) in PBS were added per well for fixation for 1 hour.

The samples were washed with 300 µl of Milli-Q water, followed by the addition of 75 µl of PR. After one-hour, excess dye was removed and washing was performed with 150 µl of a hydrochloric acid solution (Labsynth, São Paulo, Brazil) 0.01 M for 30 seconds for the removal of the dye that did not bind to the collagen.

Next, the dye was removed from the cellular layers with the addition of 150 µl of a NaOH 0.1 M solution (Cromoline® Química Fina, São Paulo, Brazil) for 30 minutes. Aliquots (100 µl) of the solutions in the wells were transferred to another culture plate. Wells containing 100 µl of NaOH 0.1 M were used as the blank. Absorbance was determined in a microplate reader (Multiskan Thermo Scientific, Shangai, China) at wavelengths of 570 nm and 630 nm. Quantification was performed by subtracting absorbance of the sample from that of the blank.

2.5. Microscopic analysis

After 48 hours of culturing, the supernatants from the wells were discarded and the cells were washed with PBS. Cells were stained with 10 µg/ml of fluorescein diacetate (FDA) (Sigma-Aldrich, St Louis, Missouri, USA), which stains viable cells green and 5 µg/ml of propidium iodide (PI) (Sigma-Aldrich, St Louis, Missouri, USA), which stains dead cells red. FDA and PI were dissolved in PBS (50 µl/well) in a live/dead assay. In the same solution, the nuclear dye 4',6'-diamino-2-phenylindole (DAPI, Israel, Sigma-Aldrich) was used at a concentration of 5 µg/ml[26].

After 10 days of culturing, Picrosirius Red was used for the quantification of collagen. Images were acquired using the Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany).

For the morphological assessment, 50 µl of biostimulator at a concentration of 10 mg/ml were suspended following the instructions of the manufacturers and placed in wells of a 96-well culture plate. After preparation, the materials were assessed with the aid of a Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany).

ImageJ Particle size was analyzed post-optical microscopy using ImageJ software (version 1.53t, National Institutes of Health). For each experimental group, 50 measurements of particle (diameter in the case of circles Ellansé®, Radiesse® and Rennova® Diamond and height and width in the irregular samples Rennova® Elleva and Sculptra®) were performed on images acquired[22].

2.6. Zeta potential

The zeta potential was measured with the aid of the Zetasizer Nano ZS90 (Malvern Instruments, United Kingdom), using dynamic light scattering. The zeta potential was obtained from the electrophoretic mobility of the particles. Measurements were performed three times, with a minimum of 12 executions for each measurement. For the determination, the materials were suspended in NaCl 1 mM at a concentration of 1 mg/ml and examined in the cuvette DTS1060 in Zetasizer Nano ZS90 equipment (Malvern Instruments, Worcestershine, UK), after preparation[22].

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). The results were submitted to analysis of variance (ANOVA) followed by the Bonferroni post hoc test for viability and collagen analyses and Tukey HSD a,b test for size and zeta potential. Viability was expressed as absorbance and also the calculation of the percentage of control. In this case, the cells that underwent no treatment were designated as the 100% value. The SPSS software programs were utilized in this study. Significant differences were established at $P < 0.05$ (*) and $P < 0.01$ (**).

3. Results

3.1. Dose-response effect of Ellansé® on cell viability and collagen synthesis by fibroblasts

(Figure 1A) displays images from fluorescence microscopy confirming the presence of live fibroblasts stained with FDA, dead cells stained with PI and nuclei stained with DAPI after two days of treatment with different concentrations of Ellansé®. Reduction in cell viability found at all concentrations ($p < 0.05$) when compared to control (saline solution in culture medium). However, no difference was found among the different concentrations (0.5, 1 and 1.5 mg/ml), with means of 0.213 ± 0.031, 0.177± 0.017, 0.170 ± 0.019 and 0.182 ± 0.012 for the control, 0.5, 1 and 1.5 mg/ml, respectively **(Figure 1B)**.

Bright field microscopy **(Figure 1C)** and quantification of staining by spectrophotometry were performed for the assessment of the biosynthesis of collagen by fibroblasts after 10 days of treatment with different concentrations of Ellansé® and PR staining. No difference was found in the synthesis of collagen among the groups ($p= 0.082$), with means of 0.295 ± 0.025, 0.290 ± 0.020, 0.318 ± 0.026, 0.308 ± 0.017 for the control, 0.5, 1 and 1.5 mg/ml, respectively **(Figure 1D)**.

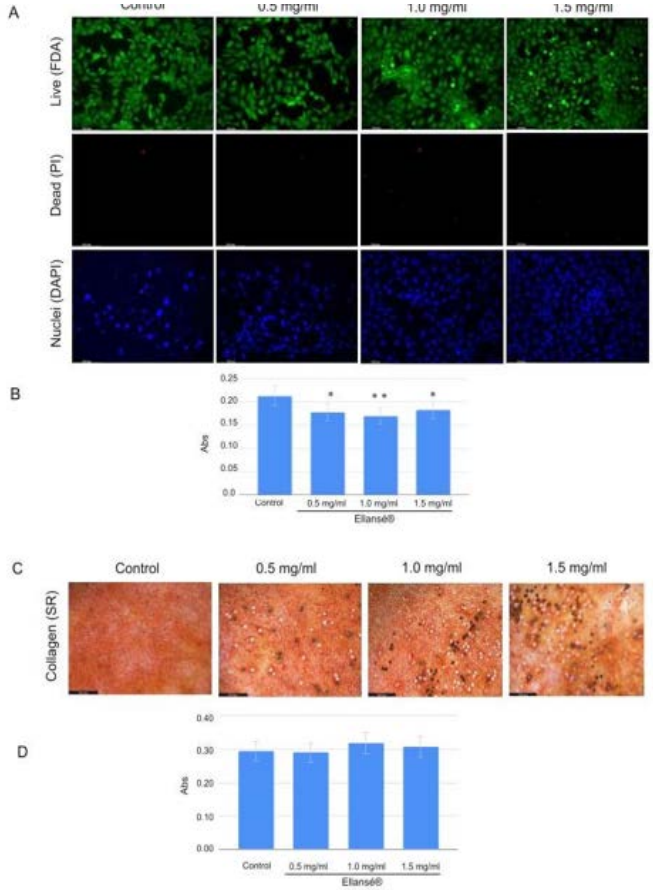

Figure 1: Effect of Ellansé® at concentrations of 0.0, 0.5, 1.0 and 1.5 mg/ml on fibroblasts.

A) Fluorescence microscopy of viable cells stained with fluoresceine diacetate (in green), dead cells stained with propidium iodide (in red) and nuclei stained with 4',6'-diamino-2-phenylindole (in blue). Bar scale = 138.8 µm. B) Quantification of viability (MTT assay) detected significant reduction in all treatments.

C) Optical microscopy indicating no difference in biosynthesis of collagen when compared to control stained with Picrosirius Red (PR). Bar scale = 316.7 µm.

D) Quantification of collagen (PR) by spectrophotometer indicating no greater formation of collagen when compared to control. A and B analyzed after 2 days; C and D analyzed after 10 days. Data are expressed as mean ± SD of the absorbance(Abs). * $p < 0.05$; ** $p < 0.01$ compared to the control, analyzed by ANOVA followed by the Bonferroni post hoc test.

3.2. Dose-response effect of Radiesse® on cell viability and collagen synthesis by fibroblasts

(nannuFigure 2A) displays images from fluorescence microscopy confirming the presence of live fibroblasts stained with FDA, dead cells stained with PI and nuclei stained with DAPI after two days of treatment with different concentrations of Radiesse®. In the qualitative assessment of viability, no significant difference ($p = 0.099$) was found between the control (saline solution in culture medium) and different concentrations of Radiesse® (0.5, 1.0 and 1.5 mg/ml), which mean (± SD) absorbance was 0.213 ± 0.031, 0.202 ± 0.014, 0.200 ± 0.020, 0.186 ± 0.007, respectively **(Figure 2B)**.

Bright field microscopy **(Figure 2C)** and quantification of staining by spectrophotometry were performed for the assessment of the biosynthesis of collagen by fibroblasts after 10 days of treatment with different concentrations of Radiesse® and PR staining. All concentrations led to a significant increase in the biosynthesis of collagen when compared to the control ($p \leq 0.05$). However, no significant differences were found among the different concentrations, with mean absorbance of 0.295 ± 0.025, 0.441 ± 0.081, 0.397 ± 0.079, 0.393 ± 0.047 for the control and concentrations of 0.5.1.0 and 1.5 mg/ml. respectively **(Figure 2D).**

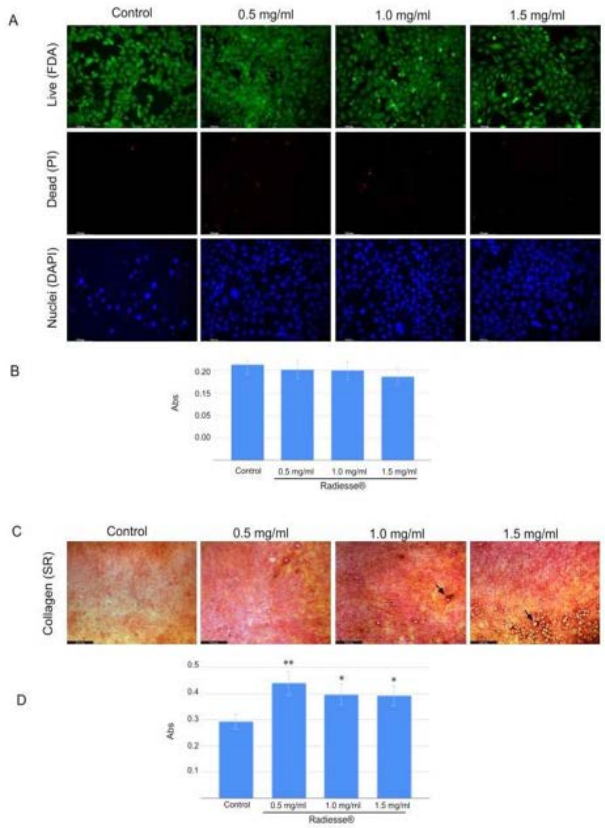

Figure 2: Effect of Radiesse® at concentrations of 0.0, 0.5, 1.0 and 1.5 mg/ml on fibroblasts. A) Fluorescence microscopy of viable cells stained with fluoresceine diacetate (in green), dead cells stained with propidium iodide (in red) and nuclei stained with 4',6'-diamino-2-phenylindole (in blue). Bar scale =

138.8 µm. B) Quantification of viability (MTT assay) revealed no significant difference among groups ($p = 0.098$). C) Optic microscopy indicating biosynthesis of collagen stained with Picrosirius Red (PR); white points indicate granule detached from plate and arrows indicate collagen synthesis around granules (brown color). Bar scale = 316.7 µm. D) Quantification of collagen (PR) by spectrophotometer indicating higher formation of collagen in all treatments with Radiesse®. A and B analyzed after 2 days; C and D analyzed after 10 days. Data are expressed as mean ± SD of the absorbance (Abs). * $p < 0.05$; ** $p < 0.01$ compared to the control, analyzed by ANOVA followed by the Bonferroni post hoc test.

3.3. Dose-response effect of Rennova® Diamond on cell viability and collagen synthesis by fibroblasts

(Figure 3A) displays images from fluorescence microscopy confirming the presence of live fibroblasts stained with FDA, dead cells stained with PI and nuclei stained with DAPI after two days of treatment with different concentrations of Rennova® Diamond. A reduction in cell viability was found at the two highest concentrations ($p < 0.05$) when compared to the control (saline solution in culture medium). However, no difference was found among the different concentrations (0.5, 1 and 1.5 mg/ml), with means of 0.213 ± 0.031, 0.200 ± 0.029, 0.179 ± 0.012, 0.178 ±0.018 for the control, 0.5, 1 and 1.5 mg/ml, respectively **(Figure 3B)**, compatible with fluorescence images.

Bright field microscopy **(Figure 3C)** and quantification of staining by spectrophotometry were performed for the assessment of the biosynthesis of collagen by fibroblasts after 10 days of treatment with different concentrations of Rennova® Diamond and PR staining. No difference was found in the synthesis of collagen in comparison to the control ($p = 0.241$) or among the different concentrations (0.5, 1 and 1.5 mg/ml), with means of 0.295 ± 0.025, 0.339 ± 0.047, 0.309 ± 0.043, 0.335 ± 0.071 for the control, 0.5, 1 and 1.5 mg/ml, respectively (Figure 3D).

3.4. Dose-response effect of Rennova® Elleva on cell viability and collagen synthesis by fibroblasts

(Figure 4A) displays images from fluorescence microscopy confirming the presence of live fibroblasts stained with FDA, dead cells stained with PI and nuclei stained with DAPI after two days of treatment with different concentrations of Rennova® Elleva (0.5, 1 and 1.5 mg/ml).

A reduction in cell viability was found at the highest concentration when compared to control (injection water in culture medium) ($p < 0.05$). Cell viability was also lower in the group with the highest concentration compared to those with lower concentrations, with means of 0.187 ± 0.006, 0.207 ± 0.023, 0.187 ± 0.019, 0.161 ±0.013 for the control, 0.5, 1 and 1.5 mg/ml, respectively **(Figure 4B)**.

Bright field microscopy **(Figure 4C)** and quantification of staining by spectrophotometry were performed for the assessment of the biosynthesis of collagen by fibroblasts after 10 days of treatment with different concentrations of Rennova® Elleva (0.5, 1 and 1.5 mg/ml) and PR staining. A significant difference ($p = 0.000$) in collagen synthesis was found at the two highest concentrations when compared to the control, with means of 0.275 ± 0.020, 0.283 ± 0.030, 0.459 ± 0.082, 0.418 ± 0.082 for the control, 0.5, 1 and 1.5 mg/ml, respectively **(Figure 4D)**.

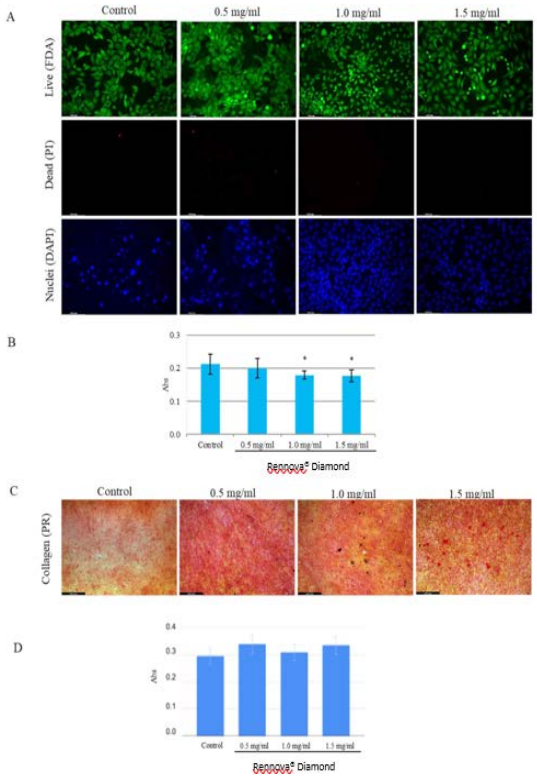

Figure 3: Effect of Rennova® Diamond at concentrations of 0.0, 0.5, 1.0 and 1.5 mg/ml on fibroblasts. A) Fluorescence microscopy of viable cells stained with fluoresceine diacetate (in green), dead cells stained with propidium iodide (in red) and nuclei stained with 4',6'-diamino-2-phenylindole (in blue). Bar scale = 138.8 µm. B) Quantification of viability (MTT assay) detected significant reduction at two highest concentrations. C) Optical microscopy indicating no difference in biosynthesis of collagen when compared to control stained with Picrosirius Red (PR). Bar scale =316.7 µm. D) Quantification of collagen (PR) by spectrophotometer indicating no greater formation of collagen when compared to control. A and B analyzed after 2 days; C and D analyzed after 10 days. Data are expressed as mean ± SD of the absorbance (Abs). * $p < 0.05$ compared to the control, analyzed by ANOVA followed by the Bonferroni post hoc test.

3.5. Dose-response effect of Sculptra® on cell viability and collagen synthesis by fibroblasts

(Figure 5A) displays images from fluorescence microscopy confirming the presence of live fibroblasts stained with FDA, dead cells stained with PI and nuclei stained with DAPI after two days of treatment with different concentrations of Sculptra®.

A reduction in cell viability was found at all concentrations when compared to the control (injection water in culture medium) ($p < 0.05$). No significant differences were found among the doses, with means of 0.187 ± 0.006, 0.164 ± 0.018, 0.157 ± 0.017,0.158 ± 0.013 for the control, 0.5, 1 and 1.5 mg/ml, respectively **(Figure 5B)**.

Bright field microscopy **(Figure 5C)** and quantification of staining by spectrophotometry were performed for the assessment of the biosynthesis of collagen by fibroblasts after 10 days of treatment with different concentrations of Sculptra® and PR staining. A significant difference in collagen synthesis was found at all concentrations when compared to control ($p \leq$

0.0.03). However, no significant differences were found among the concentrations, with means of 0.275 ± 0.020, 0.397± 0.071, 0.414 ± 0.065, 0.452 ± 0.072 for the control, 0.5, 1 and 1.5 mg/ml, respectively **(Figure 5D).**

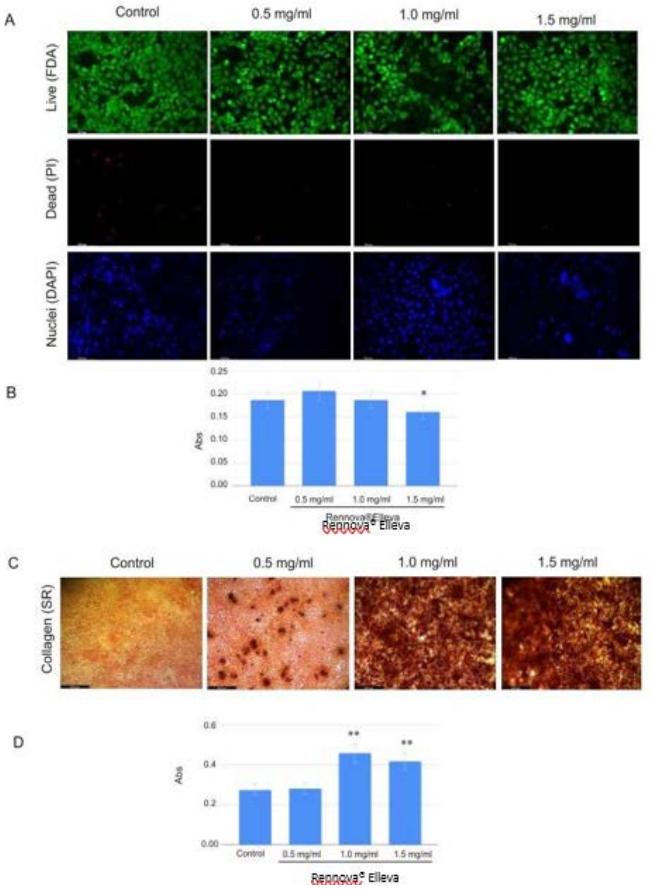

Figure 4: Effect of Rennova® Elleva at concentrations of 0.0, 0.5, 1.0 and 1.5 mg/ml on fibroblasts. A) Fluorescence microscopy of viable cells stained with fluoresceine diacetate (in green), dead cells stained with propidium iodide (in red) and nuclei stained with 4',6'-diamino-2-phenylindole (in blue). Bar scale = 138.8 µm. B) Quantification of viability (MTT assay) detected significant reduction only in group with highest concentration. C) Optical microscopy indicated greater biosynthesis of collagen in groups with higher concentrations by stained with Picrosirius Red (PR). Bar scale = 316.7 µm. D) Quantification of collagen (PR) by spectrophotometer indicating significant difference in biosynthesis of collagen in groups with concentrations of 1.0 and 1.5 mg/ml. A and B analyzed after 2 days; C and D analyzed after 10 days. Data are expressed as mean ± SD of the absorbance (Abs). * $p < 0.05$;

** $p < 0.01$ compared to the control, analyzed by ANOVA followed by the Bonferroni post hoc test.

3.6. Cell viability and biosynthesis of collagen - Comparison of products

For the purpose of comparison, the controls' absorbance values were designated as 100% and the viability of the fibroblasts treated with the biostimulants was subsequently evaluated. Viability values lower than 70% are indicative of cytoxicity.

At the highest concentrations that were examined (1.5 mg/ml), a decrease in cell viability was observed in all groups in comparison to the controls. No significant difference was identified among the five products (hydroxyapatite brands, PLLA brands or PCL; Figure 6A).

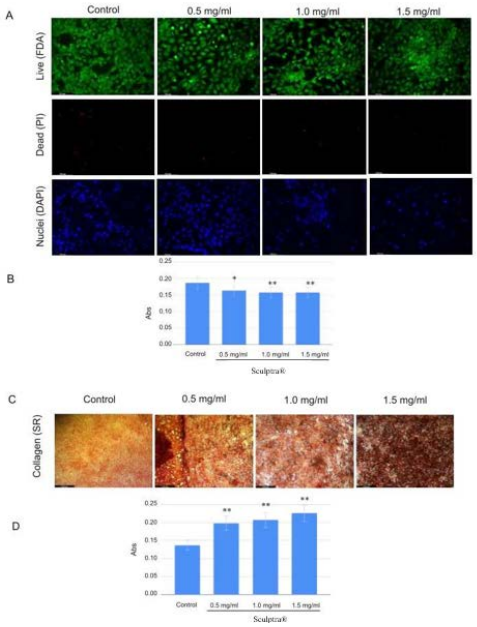

Figure 5: Effect of Sculptra® at concentrations of 0.0, 0.5, 1.0 and 1.5 mg/ml on fibroblasts.

A) Fluorescence microscopy of viable cells stained with fluoresceine diacetate (in green), dead cells stained with propidium iodide (in red) and nuclei stained with 4',6'-diamino-2-phenylindole (in blue). Bar scale = 138.8 µm. B) Quantification of viability (MTT assay) detected significant reduction in all groups. C) Optical microscopy indicated greater biosynthesis of collagen in all groups. Bar scale = 316.7 µm. D) Quantification of collagen (PR) by spectrophotometer indicated significant difference in biosynthesis of collagen in all groups when compared to control. A and B analyzed after 2 days; C and D analyzed after 10 days. Data are expressed as mean ± SD of the absorbance (Abs). * $p < 0.05$; ** $p < 0.01$ compared to the control, analyzed by ANOVA followed by the Bonferroni post hoc test.

A significant increase occurred in the biosynthesis of collagen in the Radiesse®, Rennova® Elleva and Sculptra® groups when compared to control (Figure 6B). The product with PCL (Ellansé®) demonstrated a comparable collagen biosynthesis to cells that underwent no treatment ($p = 1.000$). There is no statistically significant difference between the brands with PLLAs (Rennova® Elleva and Sculptra®). In both cases, a higher collagen level was detected compared to the control group ($p = 0.000$). In consideration of the fillers with hydroxyapatite, Radiesse® demonstrated a higher level of collagen in comparison to the control group ($p = 0.0003$), while Rennova® Diamond exhibited a comparable response to the no-treatment group ($p = 1.000$). However, no statistically significant differences were observed when comparing Radiesse® and Rennova® Diamond ($p = 0.690$).

3.7. Morphological analysis and zeta potential

As illustrated in **(Figure 7)**, optical microscopy images depict the morphology and distribution of particles suspended in the solvent indicated by the manufacturer.

Ellansé®, Radiesse® and Rennova® Diamond had similar morphology: spherical, symmetrical and uniform particles.

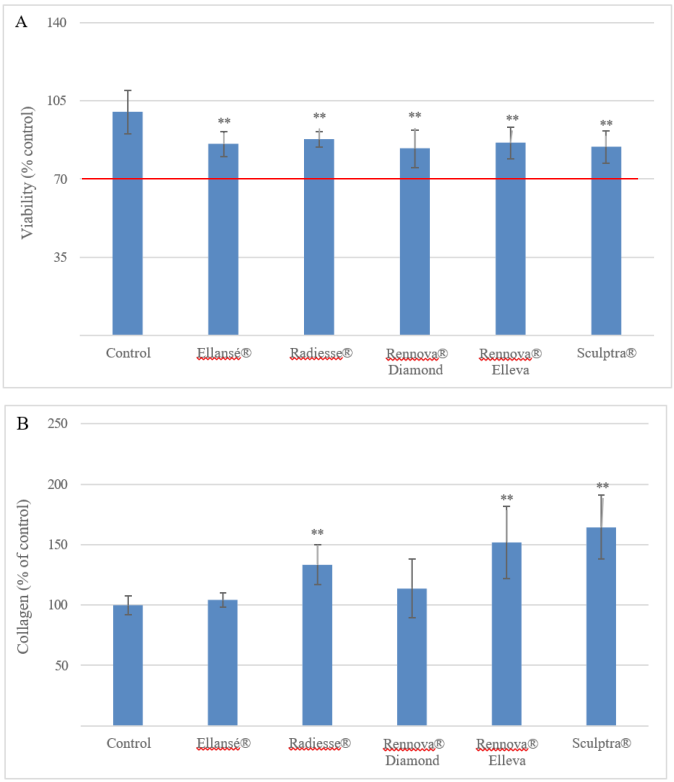

Figure 6: Evaluation of different brands of biostimulators in fibroblasts treated with a concentration of 1.5 mg/ml of the different products in comparison to the control group (100%). A) Cell viability (percentage of control after MTT assay). Compounds are classified as cytotoxic if the viability falls below the 70% red line. No significant differences were observed among the five different brands of biostimulators (ps ≈ 1).

Quantification of collagen biosynthesis stained with Picrosirius Red by spectrophotometer (percentage of control). Data expressed as mean ± SD (n= 8).

** $p < 0.01$ indicated difference from control by one-way ANOVA followed by Bonferroni post hoc test.

Rennova® Elleva (PLLA) had particles with an irregular shape, similar to snowflakes, that were asymmetrical, of different sizes and with a dark grey tone. Sculptra® also PLLA had particles with an irregular shape that were asymmetrical, of different sizes, but with rounded angles.

The statistical analyses of size conducted using ImageJ following optical microscopy indicated a statistically significant difference in the distribution of length/diameter of these particles of biostimulators with $p = 0.000$ **(Figure 7B)**. In the spherical particles (Ellansé®, Radiesse® and Rennova® Diamond) the diameter was evaluated and they present similar size. Rennova® Diamond exhibited smaller particle sizes, with values of 27 ± 5 µm. The mean and standard deviation size of the Ellansé® particles was 35 ± 6 *µm,* while the mean size of the Radiesse® particles was 31 ± 5 *µm. In* contrast, Rennova® Elleva and Sculptra® exhibited the highest degree of dispersion and size, with values of 37 ± 21 µm and 54 ± 24 µm, respectively.

Zeta potential measurements were performed to assess the surface charge of the particles. The term "colloidally stable" is employed to denote a material that has demonstrated the capacity to maintain its colloidal stability, which is defined as the absence of aggregation. This aggregation-inhibition capability is attributed to the material's zeta potential, which must possess an absolute value greater than + 30 mV or less than - 30 mV [27]. This value is considered significant, as it ensures sufficient electrostatic repulsion to impede the process of aggregation. The following zeta potentials were found for the biostimulators: Rennova® Diamond, - 2.0 ± 0.4 mV; Radiesse®, - 10.5 ± 1.6 mV; Ellansé®, - 20.1 ± 2.6 mV; Rennova® Elleva, - 40.0 ± 8.4 mV; and Sculptra®, - 53.6 ± 6.6 mV (Figure 8).

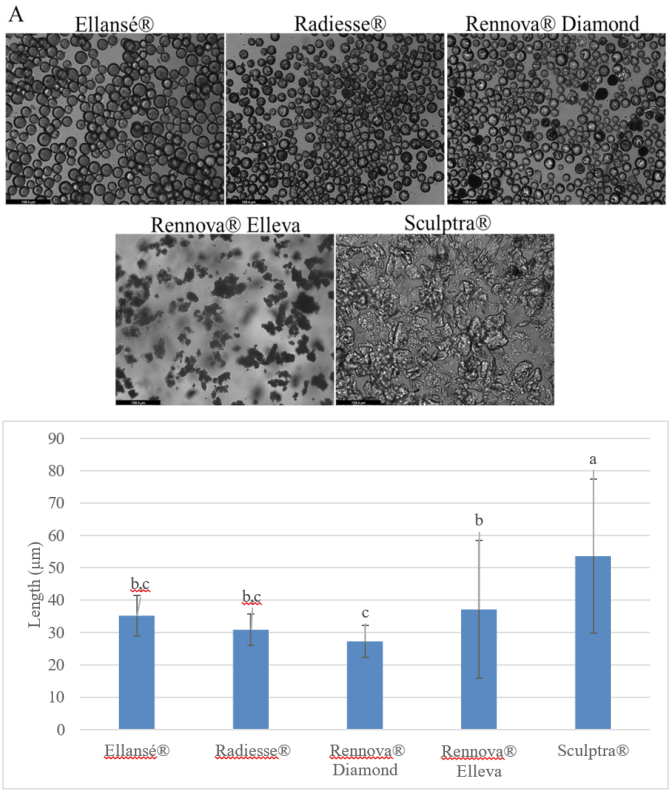

Figure 7: A) Optical microscopy with morphological aspect of Rennova® Diamond, Radiesse®, Ellansé®, Rennova® Elleva and Sculptra® biostimulators immediately after resuspension in the solvent indicated by the manufacturer (injection water for Elleva and Sculptra®; Radiesse®, Rennova® Diamond and Ellansé® dispersed in saline solution, following the recommendations of the manufacturers). The scale bar represents 158 µm. B) Graphical representation of size particles. Data expressed as mean ± SD (n = 50). Different letters indicate significant differences among means ($p \leq 0.05$) and the same letters indicate statistical equivalence ('b,c' means statistical equivalence with both 'b' and 'c') as determined by one-way ANOVA followed by Tukey's post hoc test.

4. Discussion

The process of aging skin is a multifaceted phenomenon influenced by a multitude of intrinsic and extrinsic factors. Clinically, it manifests as dryness (xerosis), wrinkles, pigmentation changes (dyschromia), thinning (atrophy) and loss of elasticity (laxity). Histologically, aging skin is characterized by epidermal thinning, a reduction in rete ridge prominence and decreased dermal thickness. At the molecular level, the structural integrity of the dermis and its extracellular matrix is compromised, contributing to the observed clinical and histological changes. Collagen production declines, while existing collagen undergoes degradation, becoming thicker, fragmented and disorganized. Additionally, the proportion of collagen III relative to collagen I increases, in contrast to the balance found in youthful skin. Elastic fibers diminish in

number and size, appearing more disordered. Furthermore, mucopolysaccharides, including hyaluronic acid, are reduced. Biochemically, key extracellular matrix cells, such as fibroblasts and macrophages, decrease in number, along with their secretion of essential growth factors like transforming growth factor beta (TGF-β)[28].

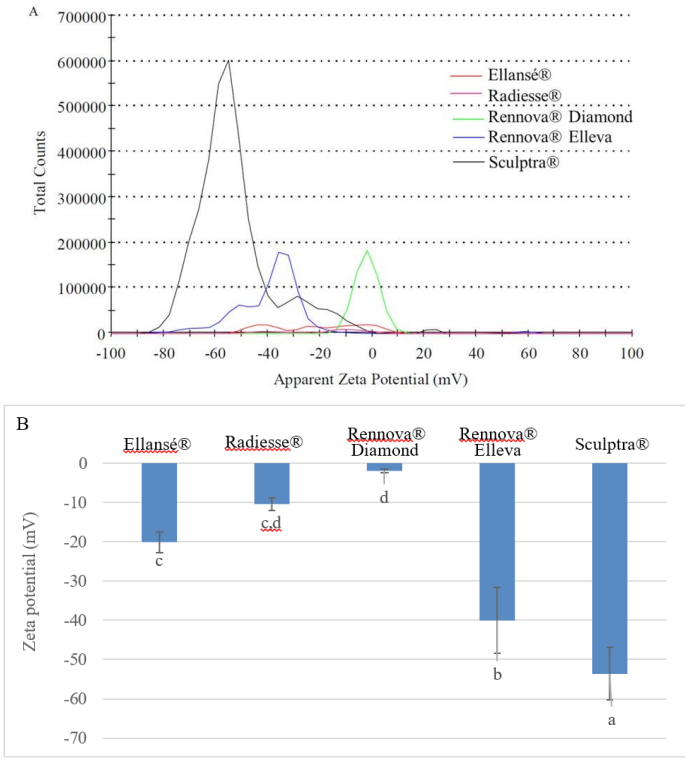

Figure 8: Zeta potential measurement of Ellansé®, Radiesse®, Rennova® Diamond, Rennova® Elleva and Sculptra® biostimulators immediately after suspension in NaCl 1 mM (zeta potential usual solvent). A) Distribution. B) Graphical representation with data expressed as mean ± SD (n = 3). Different letters indicate significant differences ($p \leq 0.05$) by ANOVA, followed by Tukey's test and the same letters indicate statistical equivalence ('c,d' means statistical equivalence with both 'c' and 'd').

For many years, a wide array of natural and synthetic materials has been employed to enhance human tissue and attain enhanced aesthetic outcomes. Among these materials, dermal fillers have emerged as some of the most prevalent treatments[29].

The aim of the present study was to investigate the action of biostimulators in terms of the viability and production of collagen by MRC-5 cell culture. No previous studies of this type comparing the most widely used biostimulators were found in the literature.

Cell viability is defined as the capacity of cells to survive, sustain metabolic activity and function normally subsequent after exposure to particular conditions, including biomaterials, pharmaceutical agents or environmental factors[30]. According to ISO 10993-5 (2009), a material is classified as cytotoxic only if its cellular absorption is less than 70% on the cell viability assay[31].

The influence of biostimulators on cells is a subject of considerable interest in the field of biological sciences. These biostimulators have been observed to modulate the activity of fibroblasts and macrophages, thereby stimulating the production of collagen. It is noteworthy that these collagen types are associated with both inflammatory and non-inflammatory processes. The response of these cells to biostimulators is highly dependent on several factors. These factors include the specific biostimulator in question, the surrounding cellular environment and the activation of diverse signaling pathways. The injection of dermal fillers instigates a regulated inflammatory reaction, which prompts the attraction of macrophages. The M1 macrophages secreted proinflammatory cytokines, such as IL-6, IL-1β and TNF-α subsequently activate fibroblasts. This acute inflammatory response is characterized by increased levels of IL-10 and a predominance of M2 macrophages. This reaction facilitates fibroblast migration towards the filler. These M2 macrophages, in turn, stimulate TGF-β production, which activates fibroblasts and initiates collagen synthesis. Other studies have indicated that fillers directly stimulate dermal fibroblasts to increase collagen production by activating the TGF-β/Smad, Akt, p38 and JNK signalling pathways. This process of collagen neoformation results in the restructuring of the skin, leading to enhanced firmness and elasticity[29].

In the present study, the biostimulators tested presented variables results in the viability of MRC-5 fibroblasts, but none exhibited cytotoxicity. According to the ISO 10993-5 guidelines, a material is deemed cytotoxic if it results in a reduction of cell viability by more than 30%, corresponding to a viability below 70% in vitro assays[31]. Radiesse® at 0.5, 1.0 and 1.5 mg/ml exhibited comparable levels of MRC-5 cell viability to the control group, which received no treatment. The results of the MTT test of cells cultured with Rennova® Diamond indicate statistical similarity of the absorbance at 0.5 mg/ml. The absorbance was statistically lower than the control at the highest doses (1.0 and 1.5 mg/ml). The Ellansé® and Sculptra® treatment produced a statistically significant non-cytotoxic reduction in viability at the three doses that were evaluated. Rennova® Elleva demonstrated a statistically significant reduction in the viability of MRC-5 cells at the highest concentration tested (1.5 mg/ml).

Although more sensitive to cytotoxicity in comparison to human primary cells, MRC-5 fibroblasts are indicated for this type of study by the American National Standard ISO 10993-5 due to the reproducible growth rates, greater availability compared to human primary cells and for being an immortalized cell line[31].

The reduction in cell viability determined by the MTT test does not necessarily mean a greater occurrence of apoptosis. The results may be explained by a greater number of cells with lower metabolic activity in the period analyzed[32].

With regards to cell viability of fibroblasts exposed to the hydroxyapatites for 24 and 48 hours at concentrations of 0.5 mg/ml and 1 mg/ml, the present results were similar to those described in the study by Courderot-Mazoyer and colleagues[33] for Radiesse®. Another study investigated Sculptra® at concentrations of 0.05, 0.1, 0.5 and 1 mg/ml for 24, 48 and 72 hours and found lower fibroblast viability at all concentrations compared to the control[34]. Similar results were found in the present investigation for both Sculptra® and Rennova® Elleva.

An improvement in cell viability prior to or together with a stimulus, such as a biostimulator, may make the cells more responsive to treatment. Thus, several studies have investigated the use of nutrients and growth factors, such as biomaterials with catechins/flavonoids and fibrin-rich plasma, for a better tissue

engineering performance[35,36]. To achieve good tissue repair or regeneration, it is important to have three factors in the treatment: nutrients/growth factors, cells (the material inserted into the adequate layer of the cellular niche) and scaffolds (matrices), which, in this case, are the biostimulators[36-38].

Total collagen in adult human dermis is represented mainly by type I collagen (80%) and type III collagen (10%)[39]. The present study only analyzed collagen produced by MRC-5 cells in the absence of inflammatory cells. Typically, such collagen is considered noninflammatory due to the lack of pro-inflammatory cells. In the initial phase of the response to injury or the introduction of a dermal filler, inflammatory cells, including macrophages and neutrophils, play a pivotal role[40]. These cells function by removing cellular debris and releasing growth factors and cytokines. These factors, in turn, stimulate the migration and proliferation of fibroblasts[41]. Subsequently, the production of collagen is initiated by fibroblasts[42]. So, this research paper was undertaken to enhance comprehension of the mechanism of action of biostimulators.

In the study conducted by González & Goldberg, Radiesse® increased the quantity of proteoglycans, which, in turn, have also have an effect on elastin. The results of the study also indicated that hydroxyapatite could induce the remodeling of components of the extracellular matrix[36,43]. In the present study, the calcium hydroxyapatite Radiesse® stimulated collagen production. In contrast, Rennova® Diamond did not increase the synthesis of collagen by MRC-5 cells when compared to the control but could have the capacity to induce proteoglycan components of the extracellular matrix.

Kim and collaborators[44] raised the hypothesis that Sculptra® could directly affect fibroblasts even in the absence of inflammation. Increasing effects were found in the expression of the gene of type I collagen detected using RT-PCR in 48 hours of incubation, suggesting the p38, Akt and JNK signaling pathways as the form of activation. The findings of the present investigation lend strength to this hypothesis and included the newest PLLA on the market (Rennova® Elleva), which, although produced using a different method. B3Homos+ manufacturing technology, an exclusive freeze- drying process of hermetically vacuum-packed PLLA microparticles, has been demonstrated to provide rapid reconstitution and a homogeneous solution within one hour. This technique has the potential to stimulate collagen synthesis. These findings question the current literature, which considers the mode of action of these biostimulators to be only a subclinical inflammatory reaction[45-47].

Ellansé®, Radiesse® and Rennova® Diamond utilize a 30% active component, with Ellansé® employing PCL while Radiesse® and Rennova® Diamond utilize hydroxyapatite. In the present study, optical microscopy images were utilized to elucidate the characteristics of the particles under investigation. There are shared characteristics of spherical particle shape, comparable size and zeta potential near zero ranges across all three fillers. The uniformity in particle morphology likely contributes to similar injection characteristics and potentially influences the distribution and integration of the biostimulators within the dermal tissue. The use of 70% carboxymethylcellulose (CMC) as the vehicle in all three products further underscores their formulation similarities. CMC is a biocompatible polymer commonly used in dermal fillers for its rheological properties and its ability to provide smooth and cohesive gel. The microscopic analysis performed in this study reinforces the observed similarities, demonstrating a narrow size and shape distribution for the particles in all three products. The absence of significant polydispersity suggests a controlled manufacturing process.

Microscopic analysis of Rennova® Elleva and Sculptra® particles revealed a notable polydispersity in both size and shape. These commercial PLLA particles sound relatively bigger in size. It appears that Rennova® Elleva is composed of larger agglomerates and smaller particles, exhibiting high contrast and dark particles. In contrast, Sculptra® appeared more translucent, with visible rounded edges. The observed heterogeneity in particle dimensions and morphologies suggests a complex interplay of factors during the particle formation process. Several mechanisms could contribute to this polydispersity. For instance, polymer chain entanglement during microparticle fabrication can result in variations in size and shape due to non-uniform packing of chains. This variability in particle dimensions may, in turn, influence the extent of the inflammatory response. The irregular morphology of Rennova® Elleva and Sculptra® particles suggests a different behavior compared to biostimulants with spherical particles, which may affect aspects such as stability, injectability and biological response.

The zeta potential analysis of surface charge revealed that all samples displayed a negative zeta potential. This negative surface charge, due to the ions in the particles structure, can influence cell proliferation and collagen biosynthesis. With regards to zeta potential value, in summary, dispersions with zeta potential values ranging from 0 ± 10 mV are classified as unstable, from ± 10 to ± 20 mV as relatively stable, from ± 20 to ± 30 mV as moderately stable and from ± 30 mV and above are classified as highly stable[27].

As the charge in the zeta potential analyses was nearly neutral, the hydroxyapatite- based biostimulators (Rennova® Diamond with mean of - 2.0 mV and Radiesse® with mean of - 10.5 mV) did not exhibit good colloidal stability. Ellansé®, with a mean value of -20.1 mV, demonstrated relative stability, while the PLLA-based biostimulators (Rennova® Elleva and Sculptra®) exhibited good stability, with mean values of - 40.0 and - 53.6 mV, respectively. The observation of a highly negative charge suggests the presence of a strong repulsive force between the particles. This phenomenon may contribute to enhanced dispersion and stability in solution[27].

A correlation between size and zeta potential with in vitro collagen synthesis could not be established, as Sculptra® presented particles with larger diameter and more negative zeta potential values and the cells treated with this biostimulator produced a high quantity of collagen. Conversely, high concentrations of collagen were also detected in fibroblasts cultured with Radiesse® and this material presented a smaller size and zeta potential. Conversely, the active component poly-L-lactic acid (in Rennova® Elleva and Sculptra®) in conjunction with calcium hydroxyapatite (in Radiesse®) has been observed to induce higher collagen concentrations. With the utilization of calcium hydroxyapatite in Rennova® Diamond as well de PCL (Ellansé®), lower levels of collagen in vitro were detected. However, it is imperative to acknowledge that the clinical results may exhibit substantial discrepancies compared to the laboratory model employed.

Although this study makes contributions with regards to the mode of action of biostimulators and the respective dose-response relationships, clinical decision-making should not be based only on the results of in vitro studies, as the inflammatory mechanism and systemic health of patients also exert an influence on clinical results.

5. Conclusion

The results demonstrated that cell viability exhibited variable responses to different products/doses and none of the products exhibited cytotoxicity. When Radiesse® alone was evaluated, no effect on fibroblast viability was observed. In the dose-response studies, reductions in fibroblast viability were found with the two highest concentrations of Rennova® Diamond, all concentrations of Ellansé®, the highest concentration of Rennova® Elleva and all concentrations of Sculptra®. In the highest tested concentration, no significant difference was found in the viability of cells treated with hydroxyapatite brands, PLLA brands and PCL. A significant increase in the synthesis of collagen by MRC-5 fibroblasts was found in the Radiesse®, Rennova® Elleva and Sculptra® groups when compared to control. The particles exhibited a size range from 27 to 54 µm, with negative net charge. Sculptra® exhibited particles of a greater size and lower zeta potential, while Rennova® Diamond displayed particles of a smaller size and near neutral zeta potential. The cellular responses did not appear to be influenced by morphology or zeta potential.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the findings of this study are available upon request from the authors.

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

Conceptualization, G.M.M. and N.M.; methodology, G.M.M. and R.P.S.; validation, G.M.M. and R.P.S.; formal analysis, N.M.; investigation, N.M., G.M.M. and R.P.S.; resources, L.R., P.P. and M.C.B.; writing-original draft preparation, N.M., G.M.M., L.R., R.P.S., P.P., C.R.B. and M.C.B.; writing—review and editing, N.M.; supervision, N.M., P.P. and M.C.B; project administration, N.M., P.P. and M.C.B.; funding acquisition, N.M., P.P. and M.C.B.; All authors have read and agreed to the published version of the manuscript. These authors contributed equally to this work.

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