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Original article

Polydioxanone powder induces collagen synthesis in fibroblast culture? – A pilot study

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Abstract. *Background:* Polydioxanone powder appears in the scenario of biostimulators as an effective and minimally invasive alternative to delay the effects of aging on the skin caused by the degradation of collagen and elastic fibers. *Objectives:* To verify, in vitro, the ability of a polydioxanone powder to induce collagen production in a culture of human fibroblasts and compare the results with the induction promoted by the PLLA powder. *Methods:* The study evaluated if CCD-1072Sk human fibroblasts in adherent cultures (ATCC CRL-2088), into eight groups (n=12) C, PLLA, PDO, PDO# organized into two subgroups PLATES A (24h) AND PLATES B (48h) if Poly-L-lactic acid-PLLA (Rennova® elleva) and powdered polydioxanone -PDO (UltraCol – Ultra-V® Medical) would stimulate an increase in collagen production by fibroblasts. The Shapiro-Wilk test was used to analyze the data's normality. To analyze the effect of the time in which the samples were analyzed (24h or 48h) and of the C, PLLA, PDO, PDO# group on collagen concentrations, we used the Two - Way ANOVA test. To identify significant differences between subgroups, we used Tukey 's post-hoc. *Results:* Variables are presented as mean and standard deviation (SD). The results showed a significant increase in collagen production from 24h to 48h in the four analyzed groups C, PLLA, PDO and PDO#. *Conclusion:* None of the biostimulators positively influenced the production of collage.

Key words: Collagen, Aging, Fibroblasts, PLLA, Polydioxanone

Introduction

Skin aging occurs due to several intrinsic and extrinsic factors. As life expectancy increases, an interest in finding ways to delay the effects of aging on the skin has grown. Skin aging corresponds to an overlapping of benign phenotypes indicative of continuous and unavoidable histological and morphological changes, caused by intrinsic factors, such as genetic and chronological influences, and by extrinsic factors, such as environmental influences. This is accompanied by the degradation of collagen and elastic fibers in the dermis, thinning of the epidermis, impairment of fibroblast function, among other alterations, and these changes impair skin integrity, wound healing, a sensory and immunological functions[1].

Different procedures are adopted in order to control the aged appearance. These range from invasive processes, such as plastic surgery, to minimally invasive ones, such as the application of artificial implants in

the hypodermis to stimulate collagen production. Surgical procedures are not always the most viable option for a cosmetic correction of an aged appearance. This is due to the time required for the procedure and postoperative recovery, the need for hospitalization and general anesthesia, the high cost, the risks involved and the unpredictability of the results. This has favored the option for minimally invasive procedures. Botulinum toxin, volumizers, implants with or without resorbable threads, ozone, platelet aggregates, photobiostimulation, collagen biostimulators, among others, have represented a growing number of procedures aimed at controlling the signs of aging[2,3,4].

Among the minimally invasive alternatives to try to delay and minimize the effects of skin aging is the use of collagen biostimulators such as calcium hydroxyapatite, poly-L-lactic acid (PLLA) and polydioxanone threads (PDO). These stimulate the proliferation of fibroblasts in the dermis that will be responsible for the synthesis of extracellular matrix proteins. Calcium hydroxyapatite and PLLA are presented in the form of a powder that is mixed with a vehicle for administration, while PDO is only available on the market so far in the form of threads, which can be smooth (mono, bi or multifilament) spiral (mono and bifilament), mesh (Matrix® wire) or barbed. Among these procedures, the dermal implantation of state-of-the-art biostimulators, such as Ellanse®, seem capable of inducing neocollagenesis and have been widely accepted and sought after in recent years[5]. Due to their biostimulant properties, they can be indicated to prevent the aging appearance of the dermis, to promote collagen formation, to restructure the pillars and contours of the face, to reduce tissue flaccidity and scars.

About 48 biostimulator implants have been approved by the FDA and those composed of calcium hydroxyapatite, polyamide, polylactic acid (PLLA) and polycaprolactone (PCL) are often used for this purpose[6-10].

Currently, polydioxanone (PDO) implants have also been studied regarding their efficacy and safety, proving to be a viable material as a collagen inducer in animal studies[11]. A study carried out in animals that aimed to identify the histological alterations and mechanisms of absorption of PDO threads and polycaprolactone (PCL), concluded that the PDO thread stimulates the proliferation of fibroblasts through the TGF-β signaling system. The study suggests that TGF-β signal transduction leads to fibroblast proliferation, which stimulates collagen formation and tissue remodeling, and that a greater surface area between the thread and the tissue induces a greater tissue response, resulting in an increase in inflammatory cells, myofibroblasts and fibroblasts, making the tissue remodeling and rearrangement effect very variable and dependent on the shape of the thread[12].

As a powdered biostimulator, PDO compounds have a quality compatible with that of PLLA and PCL in the inflammatory response and the property of forming collagen. It was demonstrated, however, that this material presents better biodegradability and a substantial decrease in roughness on the surface of the skin, when applied to animals submitted to photoaging[13].

Seeking to evaluate the differences in the functional behavior of human fibroblasts from wrinkles and aged fibroblasts from normal human skin, a culture of fibroblasts from cell lines established from two different skin samples from each of three females undergoing plastic surgery (one from a facial wrinkle and another from normal aging skin) the potential of PLLA to compensate for a reduced metabolic activity, restore migration capacity, and inhibit lactate production in wrinkle fibroblasts and normal aging skin fibroblasts. It was observed that PLLA increased collagen I synthesis, restored migration capacity and tended to decrease the production of lactate in wrinkle fibroblasts, while it only stimulated proliferation in aging skin fibroblasts and tended to improve their migration. It was concluded that the results suggest that PLLA from Sculptra® acted as a stimulus for the production of collagen in wrinkle fibroblasts and that it is suitable to correct skin depressions, such as wrinkles[14].

It was demonstrated through a histological examination after skin biopsies that were performed pre-treatment, at 6 months, 1 year and 18 months in five patients submitted to a biostimulation treatment with absorbable PDO threads, basic type, that biostimulation with PDO threads during the first 12 months post-treatment determined neocollagenesis and fibrinogenesis, that were not induced by a mechanical transduction. The new synthesized collagen

was mainly nonspecific and predominantly type I and that, when the absorption of the threads was completed, the stimulation effect also ceased, and at 18 months a complete recovery was observed with a slight increase in type I fibrous collagen[15].

In a study where the tissue response of poly-L-lactic acid (PLLA) was compared with that of powdered polydioxanone (PDO) to assess whether polydioxanone could be effective as a collagen stimulator even in powder form, which would allow it to be used in the injectable form replacing PLLA, it was observed that injections of PLLA and PDO powder induced granulomatous reactions and showed an increase in TGF-β and type I and III collagen 2 weeks after the injection, but that they decreased 12 weeks after the injection to both products. The results of the study suggested that PDO powder affects collagen growth in the same way as PLLA, proving it to be a good option for collagen formation. However, the type of collagen which formed was not investigated[16].

In another study whose objective was to evaluate the effect of PLLA (Sculptra®; Sanofi Aventis, Paris, France) on collagen synthesis and related signaling pathways, in cultured dermal fibroblasts of the Hs68 cell line, it was concluded that PLLA acts directly on dermal fibroblasts leading to a significant increase in collagen type I gene expression and protein synthesis and it was further highlighted that their results provided the first evidence that PLLA directly stimulates dermal fibroblasts to increase collagen synthesis through the activation of P38, Akt and JNK signaling pathways, influencing adjacent macrophages, which could serve as a late trigger of collagen synthesis or a limitation of the in vitro condition[17] .

We investigated whether PLLA nanoparticles were capable of inducing the same collagen synthesis effect as Sculptra® microparticles in vitro in fibroblast cultures and it was observed that PLLA nanoparticles do not stimulate collagen production in fibroblast monocultures, but that this goal can be achieved using a macrophage/fibroblast co-culture at a concentration of about 100 μg / mL of PLLA tested in vitro. Such collagen production observed in the macrophage/fibroblast co-culture was credited to a foreign body reaction in which macrophages from the environment ingested PLLA particles, which triggered the production of a cytokine (TGFβ) that stimulated fibroblast collagen production[18].

A reliable system to evaluate the reliability of collagen formation induced by biostimulators, can be conducted through the recommended protocols in cell culture. This evokes techniques that simulate natural conditions, and involves the distribution and isolation of cells specific to a physiological activity, with the aim of understanding the biochemical processes involved in this purpose. As they proliferate rapidly, the culture of fibroblasts can define an appropriate indicator to consider the formation of tissue collagen through the insertion of study variables, since it admits to understand the physiological tendency of these cells in response to different stimuli.

Fibroblasts are cells found in the dermis and are responsible for the production of collagen and elastin, which are the main fibers of the extracellular matrix. Collagen is responsible for the tensile strength of the skin and elastin provides elasticity to the skin. A decrease of both proteins with advancing age results in increased sagging and wrinkling of the skin. Thus, the neoformation of collagen and elastin can promote a rejuvenating effect on aging skin. The biostimulating effect on the skin through the stimulation of collagen production by other products such as calcium hydroxyapatite, polycaprolactone and poly-L-lactic acid is already well documented. Polydioxanone can also promote collagen neoformation when used in the form of an intradermal implant thread, differing from other substances. The use of powdered polydioxanone, however, will represent an effective and safe alternative among the products already used as a minimally invasive alternative for facial rejuvenation.

Although this is an important proposal to investigate the possibility of collagen in the skin when stimulated by biostimulators, in the researched literature, no studies were found to compare the collagenesis promoted by powdered biostimulators composed of PDO or PLLA through fibroblast cultures. Therefore, the purpose of the present study was to verify, in vitro, the capacity of a polydioxanone powder to induce collagen production in a culture of human fibroblasts and to compare the results with the induction promoted by the PLLA powder.

Materials and methods

The present study evaluated in adherent culture of human fibroblasts, cell line CCD-1072Sk (ATCC CRL-2088) obtained from the Rio de Janeiro cell bank, if two powdered biostimulators composed of polydioxanone -PDO (UltraCol – Ultra-V® Medical) and poly-L-lactic acid – PLLA (Rennova® elleva) would stimulate an increase in fibroblast collagen production when added to the culture medium.

The sample calculation was performed based on an alpha significance level of 5% (0.05) and a beta of 20% (0.20) to reach a test power of 80% to detect a minimum difference of 4 with standard deviation of 2.94 for collagen (μg/million cells) according to (COURDEROT-MASUYER et al., 2012) Thus, the sample calculation resulted in requiring 10 samples.

Descriptive statistics were used to explore and summarize the collected data. Variables are presented as mean and standard deviation (SD). The Shapiro-Wilk test was used to analyze data normality. To analyze the effect of the time in which the samples were analyzed (24h or 48h) and of the C, PLLA, PDO, PDO# group on collagen concentrations, we used the Two - Way ANOVA test. To identify significant differences between subgroups, we used Tukey 's post-hoc. To interpret the magnitude of effect of the results found, we calculated the *partial eta squared* (np^2) and we consider values up to 0.01 as no effect, up to 0.06 as a small effect, up to 0.14 as a medium effect and above this value as a large effect (Cohen, 1988)[19]. A significance level of 0.05 was adopted. All analyses were performed using the IBM SPSS Statistics 20.0 program.

The cell line CCD-1072Sk (ATCC CRL-2088) was provided by the Rio de Janeiro Cell Bank (BCRJ). The lineage of normal human skin fibroblasts was established from the skin of the foreskin of a newborn male. Cells were grown in 25cm^2 flasks using Iscove's medium Modified Dulbecco's Medium (IMDM) consisting of 4 mM L-glutamine, 4500 mg/L glucose and 1500 mg/L sodium bicarbonate and fetal bovine serum to a final concentration of 10%. 50μL of penicillin-streptomycin was also used for each 50mL of the culture medium. Cells were incubated in an appropriate atmosphere and temperature – air humidity at 95%; carbon dioxide (CO2) at 5% and temperature at 37°C. The culture medium was always replaced every three days, and the subculture was performed whenever the flasks reached confluence.

Eight 12-well culture plates were used to culture the fibroblast samples that were used in the experiment. For the statistical analysis of the results, four groups were formed, C (negative control group), PLLA (positive control group with 0.033mg of PLLA), PDO (test group with 0.1mg of PDO) and PDO# (test group with 0.033mg of PDO), each group consisting of a 12-well culture plate. The eight plates were subdivided into two subgroups of four plates identified as "Plates A" (which had their results analyzed through the ELISA test after a period of 24 hours of incubation of the culture in the presence of biostimulators) and four plates were identified as "Plates B" (which had their results analyzed through the ELISA test after a period of 48 hours of incubation of the culture in the presence of biostimulators). One of the test groups (PDO#) received an amount of product equal to that of the positive control group aiming for an equal dose.

For plating the samples that were used in the experiment, the culture flasks were subjected to trypsinization with the culture medium being removed and discarded from the culture flasks and the cell layer being rinsed quickly with Phosphate Buffered Saline (PBS) without calcium and magnesium to remove all traces of serum that contained the trypsin inhibitor. 2.0 to 3.0 mL of Trypsin-EDTA solution was added to the flasks, and the cells were observed under an inverted microscope until the cell layer was dispersed (usually in 5 to 15 minutes). 6.0 to 8.0 mL of the complete growth medium was added to the flask and the cells aspirated, which were then plated at a density of 40 x 10^3 cells per well. The plates were incubated for 24 hours in an appropriate atmosphere and temperature – air humidity at 95%; carbon dioxide (CO^2) at 5% and temperature at 37°C.

A biostimulating powder was added to the culture medium of each well of the plates. In the negative control group C no biostimulator was added, in the positive control group PLLA 0.033mg/ mL of PLLA, and in the test group PDO 0.1mg/ mL of PDO and in the test group PDO# 0.033mg/ mL of PDO. "Plates A" were incubated for 24 hours and "Plates B" were incubated for 48 hours. After this period, the type I

collagen formed was quantified using the ELISA test.

The protocol for preparing the PLLA powder was recommended by the manufacturer of the biostimulator based on poly-L-lactic acid Rennova® Elleva. Briefly, it consisted of diluting the contents of the product vial in 16ml of sterile water for injection. After adding water, the flask was shaken for 10 minutes and then left to rest for 1 hour to ensure complete hydration to obtain a uniform translucent suspension. Immediately before use, the suspension was shaken again according to the manufacturer's instructions. The PDO powder preparation protocol was also recommended by the manufacturer of the polydioxanone -based biostimulator ULTRACOL 200. Briefly, it consisted of diluting the contents of the product vial in 2mL of sterile water for injection. After adding water, the flask was well shaken to mix ULTRACOL with water and the obtained solution was put to rest for 3 hours before use. Just before use, the solution was shaken again for a better suspension, as recommended by the manufacturer.

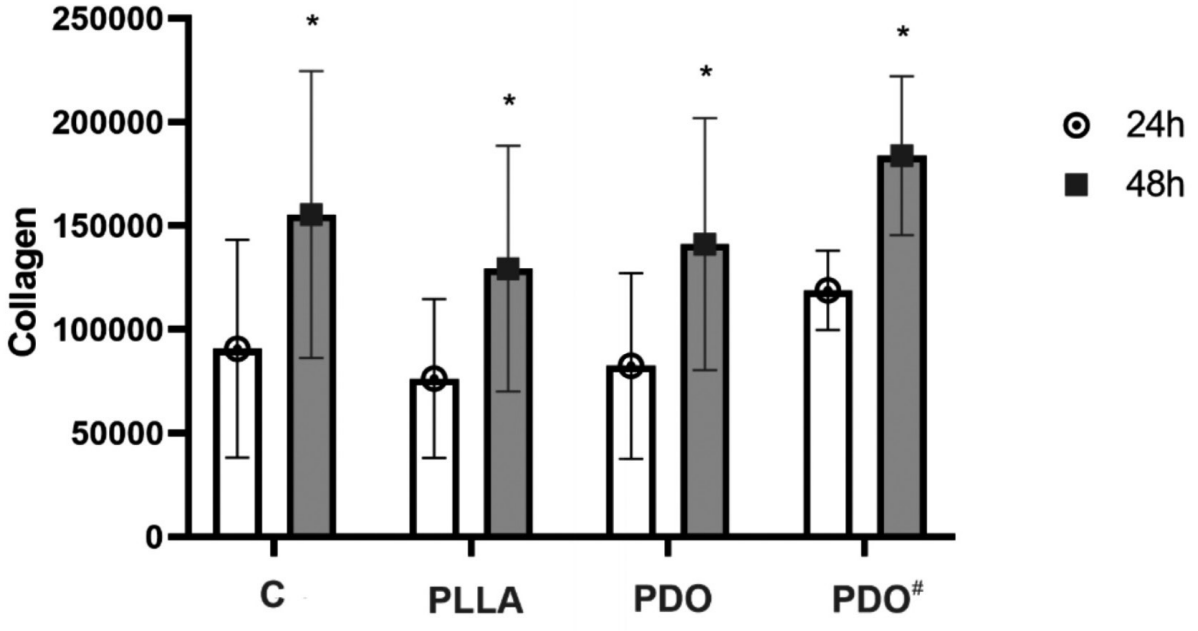
After 24 hours of incubation, the culture medium of "Plate A" was removed and mixed with protease inhibitors (10mL of EDTA and 20kl/ mL of aprotinin) to prevent protein degradation. Immediately after collection, the medium was frozen and stored at -80 °C until use for the quantitative evaluation of collagen. After 48 hours, the same procedure was performed for Plate B. Type I collagen levels were quantified using the ELISA immunoenzymatic assay.

Results

Figure 1 presents the analysis of collagen according to the times when the analyses were carried out for the different groups. We found a significant effect of time, in which all samples showed a significant increase in collagen from 24h to 48h. We did not find a significant difference between groups or interaction.

Discussion

This study is innovative in that it compares the induction of collagen production in vitro in cultured fibroblasts after cell stimulation by polydioxanone powder (PDO) and poly-L-lactic acid powder (PLLA). The results showed a significant increase in collagen



*Legend: *Statistically significant difference in relation to the 24h moment (p<0.05). Source: Ermino Souza*

Figure 1. Collagen concentration as a function of the time when the samples and groups were analyzed.

production from 24h to 48h in the four analyzed groups C, PLLA, PDO and PDO#. However, we did not find significant differences between the groups that received the biostimulator (PDO or PLLA) and the control groups, indicating that the increase in collagen was possibly independent of the direct action of the biostimulators on the fibroblasts.

In our study, we used a cell line obtained from the skin of the foreskin of a newborn, as in previous studies by Kim[a] et al., 2019[17] and by Ray & Ta, 2020[18]. It is noteworthy that although the cell lines are from the same tissue type, the samples were obtained from different individuals.

Previous studies made comparisons of these biostimulators in isolation and presented contradictory results, indicating the need for further investigations on this topic. The discussion will be developed for each biostimulator following the scientific literature produced so far.

Our study showed an increase in collagen production from biostimulation with PLLA in the test groups, but of the same magnitude as that presented by the control group. These results are contradictory to those presented by Kim[a] et al. 2019[17] which showed a significant increase in collagen production using PLLA dry powder in the human fibroblast cell line (Hs68) from 24h to 48h compared to the control group. However, some methodological differences, such as the dilution for PLLA that we used, may explain the different results found in relation to our study.

Equal magnitude responses presented by the control group and test group with PLLA were also found in previous studies. Couderot-Masuyer et al. 2012[14] used dry powder of PLLA (Sculptra®) in cultures of fibroblasts established from biopsies of wrinkles and normal aged skin of healthy patients undergoing plastic surgery procedures, with the aim of verifying the potential of PLLA to compensate for the reduction of the metabolic activity of wrinkle fibroblasts and observed that PLLA promoted a significant increase in collagen production in wrinkle fibroblasts compared to normal skin fibroblasts and in relation to the control not exposed to PLLA. This increase was verified even after 42 days of culture. These results indicate that a longer cell culture time is required to find a significant difference in collagen production between groups.

The biostimulatory effects of PLLA and PDO on collagen production did not show significant differences between the groups. Few investigations have been carried out so far with this objective. In this sense, it is worth mentioning the study by Kwon et al. 2018 [13] which compared the biostimulatory effects of Polydioxanone -PDO (trade name: ULTRACOL. Ultra V Co., Ltd., Seoul Korea), poly-L-lactic acid-PLLA (Sculptra®, Galderma Laboratories, Nestlé SA Switzerland) and Polycaprolactone - PCL (Ellansé- M®, Sinclair Pharma, Irvine, CA), in rats. The authors showed that PDO filling demonstrated neocollagenesis and inflammatory responses similar to other collagen biostimulators used in the study. The results presented in this study and our findings with cell cultures suggest a similar stimulation of collagen production between PLLA and PDO.

Ray & Ta, 2020[18] investigated whether PLLA nanoparticles were capable of inducing the same collagen synthesis effect as Sculptra® microparticles in vitro in fibroblast culture and observed that neither Sculptra® microparticles nor PLLA nanoparticles stimulated collagen synthesis in the monoculture of fibroblasts after 24 hours corroborating the results found in our study. But Ray & Ta 2020[18] reported that this goal could be achieved using a macrophage/fibroblast co-culture at a concentration of around 100 μg / mL of PLLA tested in vitro. The authors credited the collagen production observed in the macrophage/fibroblast co-culture to a foreign body reaction in which macrophages from the environment ingested PLLA particles, which triggered the production of a cytokine (TGFβ) that stimulated fibroblast collagen production. However, the short period of culture observation on the authors's part (24 hours) represents an important limitation, recognized by us in our study, with longer periods of observation being recommended in future studies.

Collagen synthesis is a complex event in vivo and involves an inflammatory reaction, with the participation of several cells, such as macrophages, which through their phagocytic activity, and release of numerous cytokines and growth factors, stimulate the proliferation of fibroblasts and collagen synthesis through these cells. Therefore, considering in vitro studies with co-cultures of cells, fibroblasts/macrophages, as in the study by Ray & Ta 2020[18], instead of isolated cultures in future studies, is a more sensible path to follow in

order to simulate the in vitro environment in vivo.

Our study also advanced with the current literature by investigating two distinct concentrations of PDO on collagen production. One group received concentrations of 0.1mg/ mL and the other 0.033mg/ mL of PDO. We found no significant differences regarding collagen production in the two groups. Based on these findings, we suggest that future studies test other concentrations or carry out a protocol for a longer period in order to compare the longitudinal effects of dosages of 0.1mg/ mL and 0.033mg/ mL of PDO on collagen production in fibroblast cultures.

Considering the concentrations of the biostimulators used in our study (0.033mg of PLLA in the positive control group), (0.1mg of PDO in one of the test groups), we are left with the question of whether the concentration of the biostimulator influences the stimulation of collagen synthesis, as similar amounts of collagen were found in both groups.

Our work is of great relevance when raising this discussion about the influence of the time in which the cells are cultivated and whether the concentration of the biostimulator exerts influence on the stimulation of collagen synthesis in cell culture, either from isolated cultures of fibroblasts or in co-cultures of fibroblasts and macrophages, offering a reference for the path to be followed, consider longer periods of observation and test different concentrations of biostimulators in future studies with cell cultures.

Although our work has presented interesting results regarding collagen production using powdered biostimulators (PLLA and PDO), it is important to recognize their limitations. First, we recognize that our results are limited to the 48h period in which the cells were observed. We suggest that future studies test this protocol using longer observation periods. Second, as far as we know, our work was probably the first to compare the effects of two different concentrations of PDO powder on in vitro collagen synthesis. We suggest that future studies use other concentrations in their experimental protocols. Finally, these works can also be complemented with investigations on the signaling pathways related to the molecular mechanisms involved in the biostimulatory action of both PDO powder and PLLA powder in the culture of cells isolated from fibroblasts or in co-cultures.

Conclusion

According to the results we found, none of the tested biostimulators positively influenced collagen production.

Disclosure of interest: The authors declare that there is no conflict of interest in the produced work. They also confirm that this work was done according to ethics and good principles.

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