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Pentoxifylline modifies three-dimensional collagen lattice model contraction and expression of collagen types I and III by human fibroblasts derived from post-burn hypertrophic scars and from normal skin

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ABSTRACT

Fibroblasts are thought to be partially responsible for the persisting contractile forces that result in burn contractures. Using a monolayer cell culture and fibroblast populated collagen lattice (FPCL) three-dimensional model we subjected hypertrophic scar and non-cicatricial fibroblasts to the antifibrogenic agent pentoxifylline (PTF – 1 mg/mL) in order to reduce proliferation, collagen types I and III synthesis and model contraction. Fibroblasts were isolated from post-burn hypertrophic scars (HSHF) and non-scarred skin (NHF). Cells were grown in monolayers or incorporated into FPCL's and exposed to PTF. In monolayer, cell number proliferation was reduced (46.35% in HSHF group and 37.73% in NHF group, $p < 0.0001$). PTF selectively inhibited collagen III synthesis in the HSHF group while inhibition was more evident to type I collagen synthesis in the NHF group. PTF also reduced contraction in both (HSHF and NHF) FPCL.

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1. Introduction

Skin wound healing involves multiple cellular processes (migration, proliferation and differentiation) allowing for the reconstruction of skin lesions. Many genetic or acquired defects can disturb the normal wound healing process leading to skin scarring due to abnormal fibroblast proliferative responses (hypertrophic scars and keloids) [1].

Hypertrophic scars (HS) after burns or other major trauma of the skin are supposed to be a dermal fibrotic disorder where red, raised, pruritic lesions distort the skin and are often associated with the formation of contractures. Scar contracture reduces the range of motion, resulting in delayed return to work and reintegration into society.

Although extracellular matrix deposition is initially increased in hypertrophic scars, regression tends to occur over

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time, and this may be in part due to changes in the distribution of proteoglycans, which are thought to be involved in the regulation of collagen fibrillogenesis. High amounts of biglycan relative to decorin are found in hypertrophic scars. The natural tendency of hypertrophic scars to regress is one manner in which they differ from keloids, although the result is always inferior to a 'normal' scar. The cytokine profile and cellular responsiveness are different for acute and regressing hypertrophic scars, and therefore these factors may also determine hypertrophic scar regression [2,3].

Once an exuberant scar is present, there are many treatments from which to choose. However, there is no universally accepted mode of treatment that results in complete and permanent hypertrophic scar or keloid amelioration. Treatments range from conservative procedures such as corticosteroid injections, pressure therapy, splinting, and serial casting to non-conservative procedures such as surgical excisions. In general, treatments are time-consuming, uncomfortable, and often incompletely successful and also associated with a high rate of recurrence [4].

Ely in 1988 first published the effects of pentoxifylline (PTF) oral therapy on giant keloids [5]. Pentoxifylline, a methyl xanthine phosphodiesterase inhibitor, is one of several agents that have been tested as a potential antifibrogenic treatment. *In vitro* studies performed on cultured dermal fibroblasts suggest that pentoxifylline may exert an antifibrogenic effect by reducing cell proliferation, by decreasing the synthesis of extracellular matrix components (including type I collagen, type III collagen, and glycosaminoglycans), and by increasing collagenase activity, due to its inhibition of tumor necrosis factor alpha (TNFalpha) [6–9]. Recent studies also suggest that pentoxifylline has a direct effect on inhibiting proliferation and FPCL of burn scars fibroblasts [10].

This study aimed to evaluate the effects of PTF on fibroblasts cell cultures isolated from samples of post-burn hypertrophic scars and from non-cicatricial skin on cell proliferation, collagen production and fibroblast capacity of contraction of a three-dimension experimental model of collagen matrix populated by fibroblasts.

2. Methods

The present study was approved by the Ethical Committee of the *Hospital das Clínicas* and the Faculty of Medicine from *Universidade de São Paulo*, Brazil.

2.1. Fibroblast cultures

Using the explant method, primary fibroblast cultures were initiated from full thickness normal human skin (NHF – $n = 10$) and from hypertrophic scars (HSHF – $n = 10$). All hypertrophic scars were over 2 years in presentation. The explants were cultured inside tissue/cells culture flasks of 25 cm^2 surface area (TPP 90025) in Dulbecco's Modified Eagle's Medium (DMEM – GIBCO 11965.092) containing 2 mM glutamine (GIBCO 25030.081), 100 U/mL penicillin, 100 μg/mL streptomycin and 25 μg/mL amphotericinB (*Antibiotic Antimycotic* – GIBCO 15249.062) in 10% fetal calf serum (FCS – GIBCO 16000.044) (nominated as $DMEM_{10}$) at 37 °C in a 5% CO_2 humidified atmosphere. These primary fibroblast cultures approached subconfluency after about 1 month of growth and were subsequently trypsinized and subcultured in the same medium with subpassage at weekly intervals.

Fibroblasts from normal skin and from hypertrophic scars were assayed for *in vitro* proliferative and biosynthetic activities between the third and the fifth passage. Assays were realized in triplicate.

2.2. Fibroblast proliferation

Fibroblast growth was evaluated in asynchronous non-confluent cultures by placing 1×10^4 freshly trypsinized fibroblasts in 1 mL of $DMEM_{10}$, into triplicate 1.96 cm^2 wells (24 multiwell plate – Falcon 35 3047) and incubation for 18 h at 37 °C in a 5% CO_2 atmosphere to permit adherence to well bottoms. After adherence, medium was removed and replaced with 1 mL fresh $DMEM_{10}$ with or without commercial pentoxifylline 1 mg/mL. Cultures were incubated for additional 96 h. Fibroblasts were then trypsinized and the number of released cells quantified by counting in a Neubauer hemocytometer chamber, with viability assessed by 0.4% trypan blue exclusion (Sigma T-8154).

2.3. Fibroblast collagen production

To assay steady-state collagen production during a 24 h period, confluent fibroblasts were cultured in the presence of ascorbic acid and 10% FCS and were assessed by ^{3}H-proline incorporation into collagenous protein. The use of confluent cultures eliminates growth-related events. Freshly trypsinized fibroblasts were plated in triplicate in 96 well microcultures (0.45 cm^2 surface area) at a near confluent density of 5×10^4 fibroblasts per well in 200 μL $DMEM_{10}$ and incubated for 48 h to produce a totally confluent monolayer of fibroblasts. The medium was then removed and replaced with 200 μL $DMEM_{10}$ containing 50 μg/mL ascorbic acid with or without pentoxifylline 1 mg/mL and incubated for an additional 48 h. Cultures were pulsed with 0.5 μCi of ^{3}H-proline (31 Ci/Mmol) for 24 h. After that period the medium containing ^{3}H-proline was removed and cells were washed with phosphate buffered solution (PBS) and breached with 200 μL of a NaOH 0.1 M solution. Scintillation cocktail was added. The ^{3}H-proline incorporation into intracellular collagen was determined by liquid scintillation. Results were expressed as counts per minute (cpm) of ^{3}H-collagen.

2.4. Collagen types I and III expression

Collagen types I and III production was assayed by immunofluorescence. 15×10^4 fibroblasts were grown over glass coverslips (13 mm diameter) in $DMEM_{10}$ with and without pentoxifylline. After 48 h fibroblasts were washed with PBS and fixed in 4% paraformaldehyde. Cells were incubated overnight at 4 °C with polyclonal anti-collagen I (1:100 in PBS) or monoclonal anti-collagen III (1:50 in PBS). Coverslips were then washed again with PBS and incubated with secondary polyclonal rabbit anti-collagen I antibodies (1:100 in PBS) or monoclonal mouse anti-collagen III antibodies (1:50 in PBS). Cells were examined by fluorescence microscopy and the

resulting images were photographed and analyzed using *Image-Pro Plus* 6.0 software. Ten high power fields were observed in each coverslip and the ratio of collagen area and total area were measured. Results of software analysis were expressed in pixels and converted to percentile (%) of fluorescent area.

2.5. *Fibroblast populated collagen lattice (FPCL) contraction*

Fibroblasts from selected subcultures were seeded in a collagen lattice and then assayed for contractile activity. Collagen lattices were prepared from type I collagen extracted from rat-tail tendons in 0.1% (v/v) acetic acid. The collagen concentration was 1.21 mg/mL. Both NHF and HSHF were trypsinized, resuspended in medium and counted using a hemocytometer chamber. A collagen–fibroblast suspension was prepared in PBS according to the *Collagen Biomaterials* protocol: 4×10^5 cells suspended in PBS (400 μL/plate) were shed into 24 well microculture plates (1.96 cm^2 surface area) followed by the addition of 100 μL of 10× PBS and 100 μL of 0.1 M NaOH. Finally, 400 μL of collagen solution was added to each plate. After 12 h, 2 mL of $DMEM_{10}$ with or without pentoxifylline 1 mg/mL were dispensed into each plate [11]. The microculture plates were photographed using a 3.2 Cybershot digital camera (Sony, Japan) 24 h and 48 h after $DMEM_{10}$ with or without pentoxifylline addiction. Photographic documentation was analyzed using UTHSCSA *Image Tool for Windows* 3.0 software where both plate and FPCL surface areas were measured. Results of software analysis were expressed in pixels and converted to cm^2.

2.6. *Statistical analysis*

A 'coortes' or follow-up study for unpaired samples was performed and a $p < 0.05$ was considered significant. The results were analyzed using generalized estimating equations (GEE).

3. Results

3.1. *Fibroblast proliferation*

As shown in Fig. 1 the exposure of fibroblasts cultures to pentoxifylline for 96 h resulted in reduction of cell numbers in both HSHF and NHF groups. In the presence of PTF the mean number of HSHF was $2.69 \pm 0.19 \times 10^4$ while in absence of PTF it was $5.82 \pm 0.30 \times 10^4$ ($p < 0.0001$). Therefore PTF inhibited HSHF proliferation by approximately 54% (Fig. 1A). The average number of NHF in the presence of PTF was $3.73 \pm 0.46 \times 10^4$ and in absence of PTF the mean number of NHF was $9.87 \pm 0.86 \times 10^4$ ($p < 0.0001$), which represents an inhibition of NHF proliferation by PTF of approximately 62% (Fig. 1B).

The anti-proliferative effect of pentoxifylline was not due to lethal toxicity on seeded fibroblasts, as the number of Trypan blue-positive dead cells in the pentoxifylline treated cultures were not statistically different from those in the untreated controls in each analyzed time point (Fig. 2). The generalized estimating equations comparison between the analyzed time points showed no statistical differences in number of dead cells ($p < 0.0001$).

3.2. *Production of collagen by fibroblasts*

As shown in Fig. 3, the inhibitory effect of pentoxifylline on human fibroblast production of intracellular collagen was remarkable in both groups: HSHF (Fig. 3A) – $1.5 \pm 0.07 \times 10^3$ cpm cpm in the presence of PTF and $3.1 \pm 0.22 \times 10^3$ cpm in the absence of PTF and NHF (Fig. 3B) – $0.9 \pm 0.03 \times 10^3$ cpm in the presence of PTF and $2.1 \pm 0.11 \times 10^3$ cpm in the absence of PTF.

Types I and III collagen expression by fibroblasts. As shown in Fig. 4, there was a statistically significant decrease in type I collagen expression by NHF. In the presence of PTF, the fluorescence ratio was $46.02 \pm 2.3\%$ and in the absence of PTF the ratio was $81 \pm 3.11\%$ ($p < 0.0001$) with no statistical significant difference observed in the HSHF group ($p < 0.0001$) (Fig. 4A).

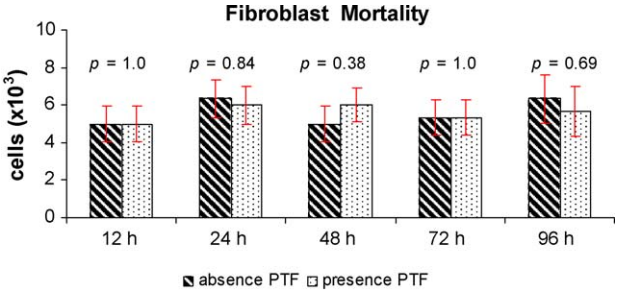

Fig. 2 – Pentoxifylline treatment did not affect the fibroblasts viability (×10^3 ± S.E.M. cells).

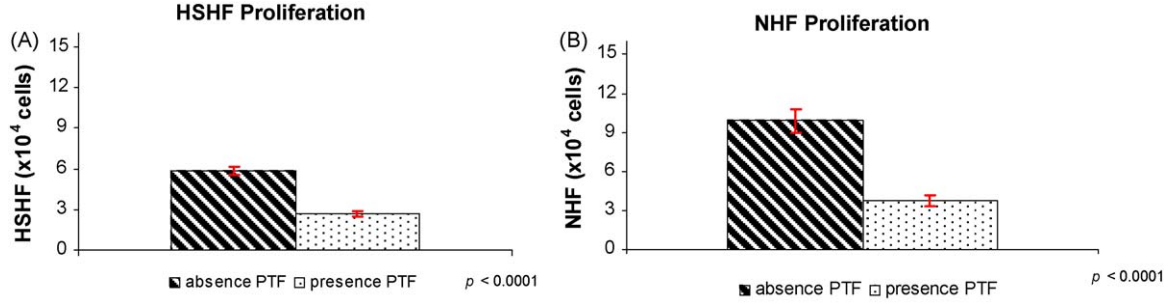

Fig. 1 – Treatment of fibroblasts cultures with pentoxifylline (1.000 μg/mL) during 96 h resulted in reduction of cell numbers in both HSHF (A) and NHF (B) (mean × 10^4 ± S.E.M.).

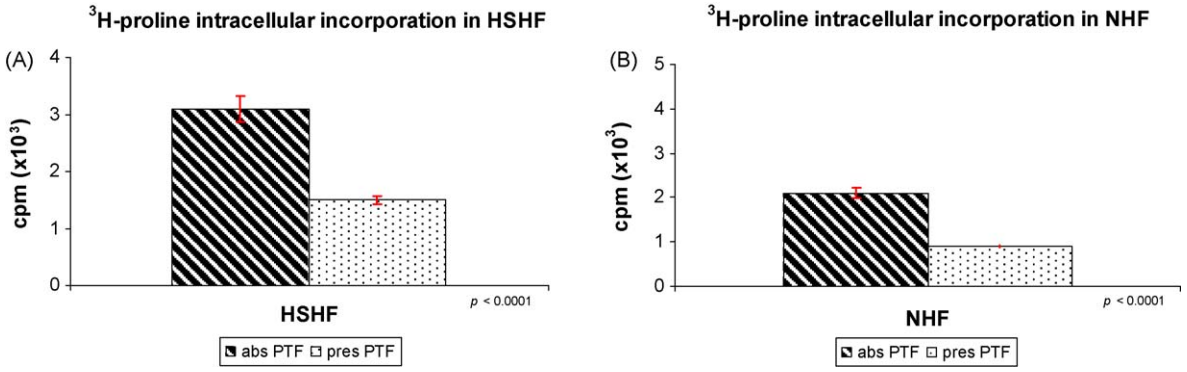

Fig. 3 – Pentoxifylline treatment reduced intracellular incorporation of ^{3}H-proline in both HSHF (A) and NHF (B) groups (mean ± S.E.M. cpm).

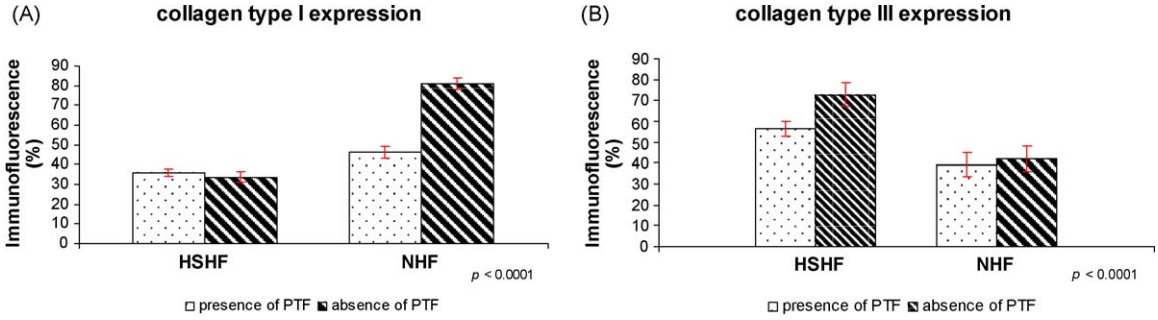

Fig. 4 – Extracellular collagens type I (A) and type III (B) immunofluorescence (% of fluorescence area).

We also observed a statistically significant decrease in type III collagen expression by HSHF. In the presence of PTF the fluorescence ratio was 56.41 ± 3.69%, and in the absence of PTF the ratio was 72.75 ± 5.7% ($p < 0.0001$) while no statistical difference in type III collagen expression was detected in the NHF group ($p < 0.0001$) (Fig. 4B).

3.3. *Fibroblast populated collagen lattice (FPCL) contraction*

The FPCL contraction assays presented a significant decrease of lattice contraction in the presence of PTF in both cell groups (HSHF and NHF). From zero to 24 h, HSHF FPCL exposed to PTF exhibited a 13% reduction in contraction while NHF FPCL exposed to PTF was inhibited by 8%. After 24 h there was no statistically significant decrease in lattice contraction ($p < 0.0001$). From zero to 48 h, HSHF FPCL exposed to PTF showed 14% inhibition while NHF FPCL exposed to PTF showed about 10% inhibition of contraction.

As shown in Fig. 5, the exposure of FPCL to pentoxifylline resulted in reduction of contraction in both HSHF and NHF groups. In the HSHF group, the area at 24 h was 1.32 ± 0.06 cm^2 in the absence of PTF and 1.52 ± 0.04 cm^2 in the presence of PTF. At 48 h, FPCL area was 1.24 ± 0.07 cm^2 in the absence of PTF and 1.44 ± 0.04 cm^2 in the presence of PTF ($p < 0.0001$) (Fig. 5A). In the NHF group at 24 h the FPCL area was 1.46 ± 0.02 cm^2 in the absence of PTF and 1.59 ± 0.01 cm^2 in the presence of PTF. At 48 h the FPCL area was 1.37 ± 0.02 cm^2

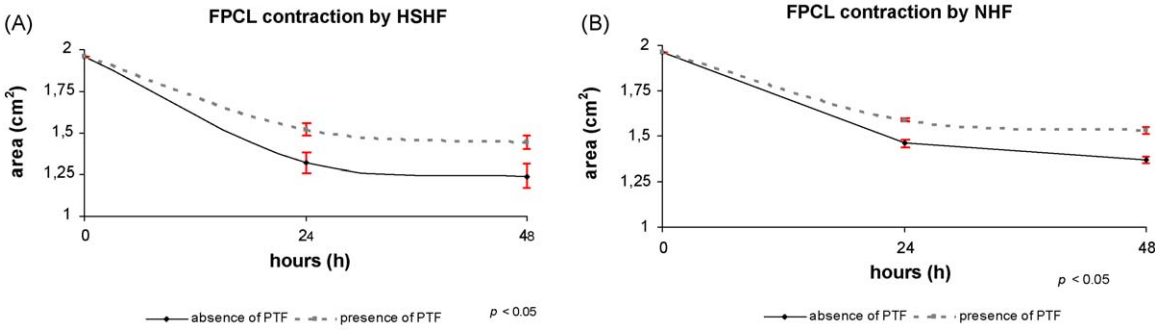

Fig. 5 – Contraction of FPCL in three different times, with or without pentoxifylline treatment. HSHF (A) and NHF (B) (cm^2 ± S.E.M.).

in the absence of PTF and $1.53 \pm 0.02\ cm^2$ in the presence of PTF ($p < 0.0001$) (Fig. 5B).

4. Discussion

When skin injury is sustained, the complement cascade leads to the release of vasoactive mediators and chemotactic factors that stimulate the migration of inflammatory cells. In the normal maturation phase, there is a decrease of stimulatory and angiogenic factors with resultant regression of hyperemia associated with early wound repair. Simultaneous collagen synthesis and degradation during normal scar maturation results in decreased nodularity and flattening of the scar [12].

The management of hypertrophic scars remains a challenge. Multiple treatments have been advocated in the past with varying degrees of success. Hypertrophic scars and keloids have been shown to respond to pressure therapy, cryotherapy, intralesional corticosteroids, radiation treatment, topical silicone or other dressings, and laser treatment [12].

Pentoxifylline is a substituted methylxanthine long used for the treatment of intermittent claudication and other conditions involving defective regional microcirculation. This hemorheologic effect of PTF is thought to be mediated by inhibition of cyclic nucleotide phosphodiesterases resulting in increased erythrocyte flexibility and production by platelets and vascular endothelial cells of prostaglandins that enhance local blood flow and promote thrombolysis. This enhancement of local blood flow, the stimulation of microcirculation, and possibly neovascularization, may be beneficial for the treatment of diseases that are caused by excessive fibrosis, as well as influence fibroblast growth *in vitro*. However, fresh hypertrophic scars display a pattern of 100–150% increase in perfusion, as has been shown in trunk and extremity scars using Laser Doppler measurements [13–15]. We believe the use of PTF *in vivo* in fresh HS might therefore cause an increase in inflammation and excessive formation of HS rather than a decrease.

Instead of using cells from immature or fresh hypertrophic scars, we selected scars over 2 years of existence to obtain fibroblasts. Normal skin was obtained from aesthetic mammoplasty.

We first aimed to evaluate the inhibitory effect on cell proliferation, synthesis of collagen, expression of types I and III extracellular collagen and FPCL model in both fibroblasts derived from hypertrophic scars and from normal skin exposed to PTF.

We observed that fibroblasts from hypertrophic scars proliferated less than fibroblasts from normal skin even in the absence of pentoxifylline. But in the presence of PTF, cell proliferation was significantly inhibited in both groups.

We also observed decreased intracellular incorporation of ^{3}H-proline by fibroblasts from hypertrophic scars and normal skin in the presence of PTF. As hidroxyproline represents 13% of collagen amino acids content we could infer that PTF inhibits collagen synthesis by skin fibroblasts.

The inhibition of type I extracellular collagen was more evident in fibroblasts from normal skin. The diminution of type III extracellular collagen was more remarkable in fibroblasts derived from hypertrophic scar. To our acknowledge this is the first study analyzing the *in vitro* effect of PTF on collagen synthesis by cells derived from post-burn hypertrophic scars. We recognize that the use of appropriate imaging software, digital photographs of high power fields, as well as the ratio of fluorescent cells are semi-quantitative assays and further experiments to characterize an inhibition of collagens types I and III expressions caused by PTF in both cell groups (NHF and HSHF) are needed.

In vitro, fibroblasts from hypertrophic scars exhibit a greater ability to contract a fibrin matrix than those from keloid or normal skin [16]. Scar contracture is dependent on myofibroblasts and these cells are found to persist in hypertrophic scars [17], an observation that has been attributed to defects in myofibroblast apoptosis [18].

Berman et al. were among the first to describe the effects of PTF in monolayer culture of normal human fibroblasts [6]. Duncan et al. showed how dermal fibroblasts exposed to PTF did not recognize the pro-collagen gene promoter nuclear factor I (NF-1) suggesting that the exposure to PTF could modify collagen synthesis in fibroblasts [9]. Berman and Duncan extended their studies to fibroblasts originated from *scleroderma*, *morphea* and keloids, also demonstrating the inhibitory effect of PTF in such cases [7].

Since described by Bell et al. [19] in 1979, the FPCL model has been wildly used to study the wound contraction phenomena [20].

According to Kamamoto et al., FPCL contraction is due to the interaction between fibroblasts and surrounding collagen fibrils. One possible explanation for this phenomenon of contraction might be the organization of the fibers and not the increase in cell number. These authors also observed a maximum contraction of FPCL 48 h after gel polymerization [21]. In the present study we have observed that the maximum contraction occurred during the first 24 h. Despite FPCLs were still visually contracting; the GEE statistical analyze showed no difference in FPCL areas between 24 h and 48 h after polymerization in both HSHF and NHF lattices. The percentiles of contraction of NHF FPCL found in our study were the same as the ones reported by Kamamoto et al.

Rawlins et al. in 2006 used *in vitro* PTF exposure of fibroblasts from mature burn scars to demonstrate an inhibitory effect of PTF on fibroblasts proliferation and contraction of FPCL [10].

The data obtained in these experiments indicate that some concepts about the proliferative and contractile phases of wound healing might have to be revised. Most specialists would deem the fibroblasts to be the sole culprits of wound contraction and collagen re-arrangement. Further studies confirming mechanisms of action and clinical use of PTF in hypertrophic scars are necessary but, according to our findings, we strongly suggest that PTF may be an alternative for treatment of post-burn scars.

5. Conclusion

In vitro exposure to pentoxifylline of fibroblasts derived from hypertrophic scars and normal skin resulted in inhibition of both intra and extracellular collagen synthesis. Moreover, *in*

vitro exposure to pentoxifylline reduced fibroblast proliferation without lethal toxicity. The specific activity of free proline intracellular pools following exposure to pentoxifylline was decreased and the 3D model presented less contraction in the presence of PTF.

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