In Vitro Effect of Triamcinolone and Platelet-rich Plasma on Lateral Epicondylitis-derived Cells

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Research article

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Abstract

Background: Lateral epicondylitis (LE) is a common musculoskeletal condition. However, the treatment of LE is still controversial. The present study evaluated the production of inflammatory cytokines by lateral epicondylitis-derived cells and compared the anti-inflammatory effect of triamcinolone acetonide with platelet-rich plasma (PRP) on their production in cell culture.

Methods: Third passage cells from primary cultures of lateral epicondylitis were assessed for the production of the cytokines IL-1**β**, IL-6, IL-8, IL-10 and TNF-**α** by immune-enzymatic assay (ELISA), after treatment with 1, 10 and 100 mM triamcinolone compared to untreated controls at the time points 6, 12, 18, 24, 48, 72 and 96 hours, and to PRP at 48, 72 and 96 hours.

Results: The cytokines IL-6 and IL-8 were produced in high concentrations by lateral epicondylitis cells. Triamcinolone induced a significant decrease in the production of IL-6 and IL-8 at 48, 72 and 96 hours, and at 12 hours for IL-8. The PRP group produced significantly higher levels of IL-8 than the control group, at 96 hours. There was a significant increase in IL-10 production with the use of 100 μM triamcinolone at 48 hours, compared to controls. The production of IL1-β and TNF-α was very low and did not change when the cultures were treated with triamcinolone or PRP.

Conclusion: Our results demonstrated that IL-6 and IL-8 plays a role in the pathogenesis of lateral epicondylitis. Triamcinolone inhibited the production of the production of IL-6 and IL-8 by lateral epicondylitis-derived cells and PRP induced an increase in IL-8 levels compared to controls.

Introduction

Tennis elbow or lateral epicondylitis (LE) is a common musculoskeletal condition that affects 1–3% of the adult population [1]. Men and women are equally affected, and it occurs in productive age groups, between 35 and 50 years of age. As it is a debilitating condition, associated with high morbidity, often leading to prolonged absence from work. The costs associated with LE are enormous, reflecting on the loss of productivity at work and health care costs [2].

Traditionally, LE is considered to be the result of recurrent mechanical overuse or overloading at the lateral elbow whereby the ability of the tendon to repair itself is overwhelmed and ultimately fails. Subsequently, this leads to microscopic tears of the tendon and an immature, abnormal reparative response [3].

Mechanical stress on the tendon with a constant frequency, intensity and duration leads to molecular changes in it, leading to the release of pro-inflammatory cytokines, which can be produced by several cell types, such as tendon fibroblasts or even chondrocytes [4, 5]. These cytokines activate intracellular signalling pathways for the release of inflammatory mediators, which leads to the degradation and degeneration of the tendon matrix [6, 7]. This continuous degeneration may result from the action of

inflammatory cytokines and mediated by fibroblasts in the injured connective tissue itself, even in the absence of inflammatory cells at the injury site [7, 8].[7,8]

Among the inflammatory cytokines, the interleukins IL-1β, IL-6, IL-8, IL-10 and TNF-α are the most studied regarding the development and progression of tendon diseases, as well as in healing and exercise response [4, 5, 8]. They are released by stromal and immunoregulatory tendon cells in the presence of tissue damage and mechanical stress, altering the phenotype of local cells [4]. The persistence of chronic inflammation results in excessive and inadequate production of protein matrix, with consequent fibrosis [5].

There is no defined treatment for LE [9], with a large number of therapeutic options, such as the use of corticosteroids, which is the most traditional treatment, or platelet-rich plasma (PRP), an autologous plasma preparation enriched with a higher platelet concentration than is normally contained in whole blood.

The therapeutic use of PRP is based on the fact that a high concentration of platelets is able to supply and release supraphysiological amounts of growth factors and cytokines and provide a regenerative stimulus, leading to repair with a low potential for fibrosis [10, 11]. The effect of almost all the growth factors present in PRP is to stimulate the collagen synthesis, angiogenesis and chemotaxis, although these two latter effects are known to be antagonistic to the desired result in the treatment of LE and other inflammatory conditions [12]. There are reports that a single injection of PRP is able to keep the patient without symptoms of LE for up to one year, while the injection of corticosteroids shows the same result in only 51% of cases [13, 14]. It is assumed that this more long-lasting effect of PRP is due to the remodelling of new tendon, which can persist and to respond better to mechanical trauma [10].

The present study aimed to evaluate the production of inflammatory cytokines in LE-derived cells in culture, comparing the effect of two widely used LE treatments, i.e. the classic use of corticosteroids versus the use of PRP.

Methods

Our Institutional Ethical Committee approved the study protocol (IRB number 2.903.373) and informed consent was obtained from all donor patients.
This was an *in vitro* study using cultures of LE-derived cells, obtained from six patients, with mean age of 44.8 years (minimum of 35 years, maximum of 54 years), equally distributed according to sex. The inclusion criteria were adults with LE diagnosed by clinical and imaging examination (simple radiography and ultrasound) in phases VI and VII according to the Nirschl clinical classification of LE.[15] Exclusion criteria were the presence of other associated orthopaedic or rheumatological diseases and percutaneous or surgical treatment for LE within less than six months.

The study material was obtained during the surgical procedure to treat the condition. The same surgeon, following a standard surgical technique, collected all samples.

Cell culture: Under sterile conditions, in a laminar flow hood, the samples were cut into small fragments of approximately 1 mm^2, which were collected into cell culture flasks, containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Grand Island, NY, USA) supplemented with 5 mL/L of TC Minimal Eagle vitamins (Sigma-Aldrich, St. Louis, MO, USA), 0.01 U/ml recombinant human insulin (Gibco, Grand Island, NY, USA), 15 µg/mL glutathione (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/mL penicillin, 40 µg/mL gentamicin, 2 µg/mL amphotericin-B (Gibco, Grand Island, NY, USA) and 20% foetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The culture flasks with the explants were kept in an incubator at 37ºC, with 5% CO_2, in a humid atmosphere.

The supplemented DMEM/F12 nutrient medium with 20% FBS was added every three days, until the cultures reached semi-confluence, when cells were sub-cultured until the third passage.

PRP preparation

During the surgical procedure, 8.5 mL of peripheral blood was collected by puncture of the anterior cubital vein of the LE patients, in vacutainer tubes containing sodium citrate as an anticoagulant and immediately taken for the processing of the PRP, following a previously described protocol for obtaining leukocyte-poor PRP [16–18]. Samples were frozen at -80^oC until use.

Cytokine study

Initially, the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was performed in order to establish the ideal non-cytotoxic triamcinolone concentrations to be used in the study on inflammatory cytokines. The analysis of this study showed a decrease in cell viability with the lowest triamcinolone concentration (0.001 µM), thus this concentration was excluded and concentrations for the following study were defined as 0.01, 0.1, 1, 10 and 100 µM (supplementary Fig. 1).

A cytokine kinetic analysis was also carried out to verify the best evaluation times and triamcinolone concentrations to be used in the study. In this study, the cytokines produced by the PRP group were measured from 12 to 48 hours, in which an increase in IL-8 levels was verified only after 24 hours (supplementary Fig. 2). Therefore, the study proceeded with the analysis of the PRP group only in later time points, from 24 to 96 hours.

After these considerations, $5x10^3$ third passage LE cells were seeded in 96-well culture plates and maintained for 24 hours in a humidified incubator at 37^oC with 5% CO_2 to allow cell adherence. The triamcinolone group was exposed in triplicate to triamcinolone acetonide at concentrations of 1, 10 and 100 µM (which had led to an effective decrease in the production of cytokines in the previous cytokine kinetics study). The control group was exposed only to complete nutrient medium. The cultures were kept in a humidified incubator at 37^oC with 5% CO_2 for 6, 12, 18, 24, 48, 72 and 96 hours. At 48, 72 and 96 hours, the effect of triamcinolone was compared to PRP treatment.

The supernatant conditioned culture medium, which was in contact with the cells during each time point after exposure to triamcinolone and PRP, was collected and analysed to determine the concentration of inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA). Commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to quantify IL-1β, IL-6, IL-8, IL-10 and TNF-α levels in the studied samples. All assays were performed according to the manufacturer's instructions. Samples in which cytokine levels were estimated to be below the sensitivity of the assay were set as equal to the sensitivity of the assay and those with concentrations at levels above standard curve were diluted and re-assayed. The assays readers were performed in an ELISA automatic reader (Epoch-BioTek, Winooski, VT, USA), at wavelength of 492 nm. The concentrations of cytokines in the conditioned culture medium were calculated on the standard curve obtained with different concentrations of the recombinant human cytokines of interest and tests were performed to determine the inter- and intra-assay variability.

Statistical analysis

The results were submitted to statistical analysis using the two-way ANOVA test and Sidak multiple comparisons test, with a significance level of 5%, performed using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Primary cell culture

After 7 days in culture, spindle cells started to migrate from explants, began to proliferate and reached confluence in an average of 21 days, when they were detached for the first passage. The cells were cultured until the third passage, when they were subjected to MTT assays and exposure to triamcinolone and PRP.

Cytokine study

The cultures from control groups that were not exposed to any of the treatments showed a high production of IL-6, which rose quickly after 12 hours, requiring dilution up to 1:25 for the detection of measurable IL-6 levels (Fig. 1). The minimum detectable levels in the IL-6 and IL-8 assays were 143.29 pg/mL, 162.62 pg/mL and zero for IL-10, IL-1β and TNF-α, respectively. Intra and inter-assay variability remained < 10.0% for all cytokines.

Triamcinolone, at the three studied concentrations, led to a significant reduction in IL-6 production by LE cells after 48 hours ($p < 0.05$), 72 hours ($p < 0.001$) and 96 hours ($p < 0.0001$), compared to controls (Fig. 2). Comparing triamcinolone to PRP, IL-6 levels were significantly lower with the use of triamcinolone in all concentrations, at 72 hours and 96 hours ($p < 0.05$ for all concentrations) (Fig. 2). The cultures exposed to PRP showed no difference in IL-6 production compared to controls.

Regarding the production of IL-8, there was also a significant reduction with the use of triamcinolone, compared to controls at times 12, 48, 72 and 96 hours ($p < 0.05$ for all concentrations) (Fig. 2). Compared

to PRP, the production of IL-8 by triamcinolone groups was significantly lower than the PRP group, for all the tested concentrations, at all time points ($p < 0.05$). The production of IL-8 by the PRP group was significantly higher than the control group ($p = 0.01$), at the time point 96 hours.

The production of IL-10 showed a significant punctual time-dependent increase ($p = 0.02$) with 100 μM triamcinolone in 48 hours, in comparison to unexposed controls (Fig. 2). The cytokines IL1-β and TNF-α showed no significant changes comparing to the unexposed controls when the cultures were treated with triamcinolone or PRP (Fig. 2).

Discussion

Our results demonstrated that cultured LE cells produce IL-6 at increasing levels, even without any external stimulus, reaching high values after 12 hours. IL-8 was also produced by LE cells, but at lower levels than with IL-6. When exposed to triamcinolone, these cells showed a significant reduction in IL-6 and IL-8 production, compared to unexposed controls and PRP. We also observed that the PRP significantly increased the levels of IL-8 at 96 hours time point, compared to controls.

The present study aimed to compare the traditional treatment, based on corticosteroids, with PRP. Intralesional steroid injection has the advantage of a low cost, is easily acquirable and has practically no side effects [19, 20], except the inherent risks related to the injection, which are the same as those for PRP.

PRP is a relatively recent treatment, with high cure rates [13, 21]. However, the response to PRP occurs only after the sixth month of application, the cost is higher than triamcinolone due to processing [13, 22] and it requires a -80^{o}C freezer for storage, if not immediately used [18].

The current study found high IL-6 levels produced by the LE cells, possibly related to the development and progression of this condition, as it is a cytokine involved in tendinopathy in humans [23–25]. IL-6 can influence several cell types, with multiple biological actions through its unique receptor system. It usually acts as a pro-inflammatory cytokine, involved in the positive regulation of inflammatory reactions and in the pain process, but IL-6 can also act as an anti-inflammatory cytokine depending on the circumstance, through the activation of its soluble receptors. IL-6 is produced by T-cells, B-cells, monocytes, fibroblasts, keratinocytes, endothelial, mesangial cells, adipocytes, some tumour cells and also by tenocytes [8].

LE cells also secreted IL-8, a potent chemokine, also with proven inflammatory involvement in tendinopathies. IL-8 plays a key role in neutrophil-mediated inflammation, which leads to cartilage destruction and bone damage [26].

PRP led to an increase in IL-8 levels compared to controls at 96 hours time point, suggesting a pro-inflammatory effect. Thus, triamcinolone can be considered more effective than PRP in reducing inflammation in the first 96 hours of treatment. The use of triamcinolone was effective at the three studied concentrations, also leading to a reduction in IL-8 levels, when compared to controls and PRP.

IL-10 showed a significant time-dependent punctual increase by 48 hours with 100 μM triamcinolone; concomitantly, there was also an increase in IL1-β at this time point, which could explain the elevation of IL-10, since it is an anti-inflammatory cytokine that regulates the intensity of inflammation [27]. It may have been produced in order to control the rising levels of IL1-β. After this time point (48 hours), IL-1-β levels gradually decreased. Although this increase in IL1-β was not statistically significant, it was probably able to stimulate the IL-10 production by LE cells.

Despite the superior results of corticosteroids on PRP in reducing the inflammatory cytokines of LE, they are not exempt from undesirable effects. It has been shown that triamcinolone 0.1 mg/mL leads to a decrease in the viability of rotator cuff cells when exposed for 7, 14 and 21 days, due to apoptosis [28]. Therefore, treatment with corticosteroids requires caution, and it is not recommended to use it with repetitions in short time intervals.

The PRP used in the present study was processed according to the protocol for the obtaining leukocyte-poor PRP [16, 17], since leukocytes, despite having important functions in tissue repair and providing protection against infectious agents, also have pro-inflammatory actions and immunological effects that can result in undesirable and opposing effects in healing, leading to increased inflammation due to pro-inflammatory cytokines [16, 29]. However, even this PRP preparation caused intense and significant production of IL-8 when compared to controls.

It is known that PRP has in its composition IL-6, IL-8, IL1-β and TNF-α [30, 31]. The development of a PRP free of pro-inflammatory factors, especially VEGF, could have provided better results [30]. Clinical studies comparing the use of PRP with triamcinolone confirm our results, with an initial advantage for the corticosteroid group and gradual improvement for the PRP group, with significant improvement in the PRP group only after six months [32]. However, according to the natural history of LE, it can evolutes with spontaneous improvement in six months. Another difficult that we found was the lack of standardization for the PRP production, which makes it difficult to compare with studies in the literature, as PRPs are very heterogeneous and qualitatively very different, with no strong evidence regarding the ideal preparation, dosage and efficacy [31, 33, 34].

The late effect of PRP in the treatment of LE, which can be up to six months, also limits the assessment of the PRP effectiveness, both in *in vitro* and *in vivo* studies. In *in vitro* studies, the maintenance of the cells in culture after exposure to PRP for up to six months is not feasible, since the cells will lose their phenotypic characteristics and the senescence of the cultures will invariably result in the loss of proliferation capacity, followed by apoptosis. *In vivo* studies also have limitations, due to the possibility of spontaneous remission of LE in most cases, not allowing for an assessment of whether the cases that were resolved resulted from the late effect of PRP or if resolution resulted from the natural course of the disease.

Conclusion

The present study shows the importance of IL-6 and IL-8 in the pathogenesis of LE, reinforcing the inflammatory feature of this condition. In addition, our results confirm the anti-inflammatory effect of triamcinolone, in contrast to that observed with the use of PRP, which triggered an increase in the production of IL-8 by LE cells *in vitro*.

Abbreviations

LEE
lateral elbow epicondylitis; PRP:platelet-rich plasma; IL-1β: interleukin-1 beta; IL-6:interleukin-6; IL-8:interleukin-8; IL-10:interleukin-10; TNF-α: tumour necrosis factor alpha; PDGF:platelet-derived growth factor; TGF-β:transforming growth factor beta; VEGF:vascular endothelial growth factor, EGF:epidermal growth factor; bFGF:basic fibroblast growth factor; IGF-1:insulin-like growth factor; MTT:3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide).

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee of the Botucatu Medical School, Brazil (ERB # 2.903.373). Informed consent was obtained from all participants.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing of interests

The authors declare they have no conflicts of interest.

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Author contributions

MEMV performed the surgeries and collected the LEE explants; MMHV, CAR, MGS performed the experiments; MEMV, MMHV, SAS conceived and designed the study; MMHV, CAR performed statistical analysis; MEMV, MMHV, SAS wrote the manuscript. All authors read and approved the manuscript.

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Figures

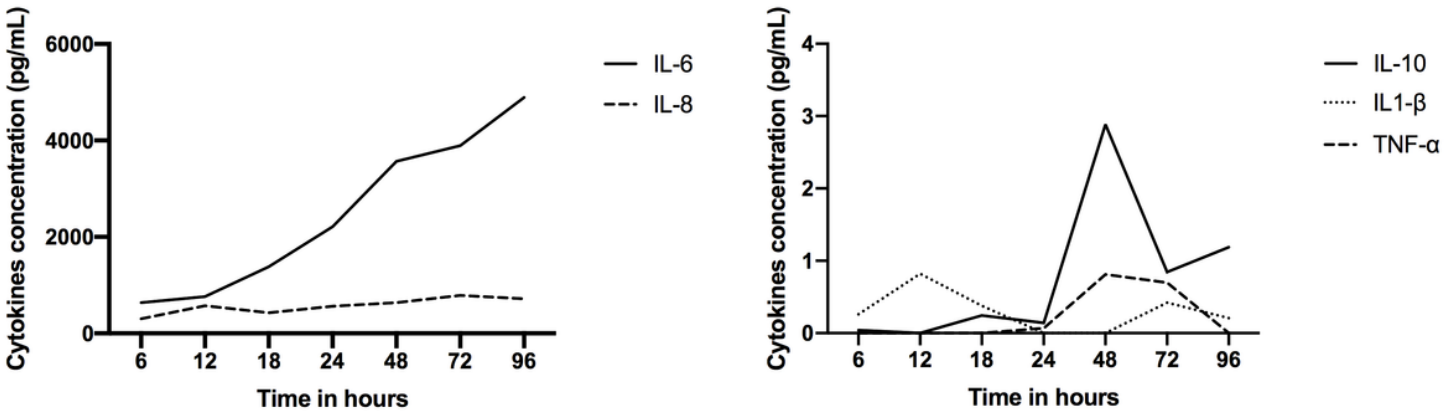

Figure 1

Levels of the cytokines IL-6, IL-8, IL-10, IL1-β and TNF-α in pg/mL produced by control group cultures at 6, 12, 18, 24, 48, 72 and 96 hours in the study on cytokine production kinetics. Levels were determined by ELISA, without exposure to triamcinolone or PRP. n = 6 donors. IL, interleukin; TNF, tumor necrosis factor.

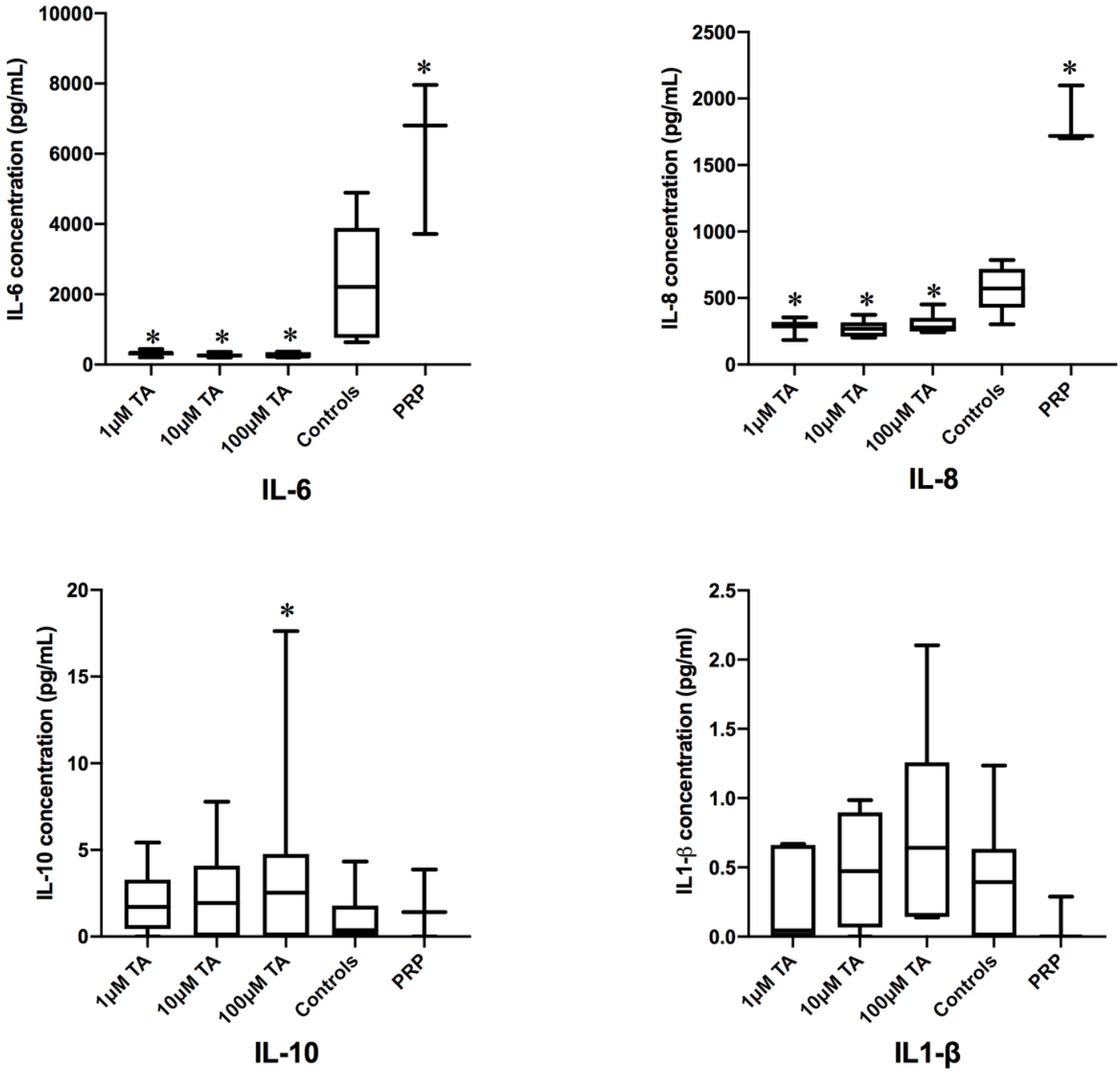

Figure 2

IL-6, IL-8, IL-10 and IL1- β levels in pg/mL produced by LE-derived cells exposed to 1, 10 and 100 μM triamcinolone acetonide (TA), measured at different time points, compared to PRP and controls, determined by ELISA. TA led to a significant decrease in IL-6 and IL-8 levels in all the studied concentrations, compared to controls and PRP. The production of IL-8 by the PRP group was significantly higher than the control group, at all studied time points. IL-10 levels showed a significant punctual increase with 100 μM triamcinolone at 48 hours, in comparison to controls. IL1-β underwent no

significant changes when the cultures were treated with triamcinolone or PRP. n = 6 donors. IL, interleukin. *p < 0.05 versus Controls.

Supplementary Files

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