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Effect of ScLL and 15d-PGJ$_2$ on viability and cytokine release in LPS-stimulated fibroblasts: an in vitro study

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Abstract: This study evaluated the effect of a cyclopentenone-type PG, 15-Deoxy-$^{\Delta 12,14}$-PG J2 (15d-PGJ$_2$), and lectin (ScLL) on the viability of human gingival fibroblasts (HGFs), and on IL-6 and TGFβ-1 release by these fibroblasts, stimulated with lipopolysaccharide (LPS). HGFs were stimulated with LPS 10 μg/ml and treated with 15d-PGJ$_2$ 1 and 2 μg/ml, and ScLL 2 and 5 μg/ml, for 1 and 3h, and then evaluated for viability by MTT assay. Supernatant was collected to detect IL-6 and TGFβ-1 release, by ELISA. Positive control was cells kept in Dulbecco's Modified Eagle's Medium, and negative control was those kept in LPS. Data were analyzed by ANOVA and Dunnett's test ($\alpha = 0.05$). No significant difference was found in viability among experimental groups at 1h ($p > 0.05$). Percentage of ScLL 5 μg/ml viable cells was similar to that of positive control at evaluated periods ($p > 0.05$), whereas the other groups had lower levels than the positive control ($p < 0.05$). IL-6 release was statistically higher for ScLL 5 μg/ml and 15d-PGJ$_2$ 2 μg/ml at 1h, compared with the other treated groups and positive control ($p < 0.05$). No significant differences were found among the groups at 3h ($p > 0.05$), except for ScLL 2 μg/ml and 15d-PGJ$_2$ 1 μg/ml, which showed lower IL-6 release compared with that of negative control ($p < 0.05$). No significant difference was found among the groups for TGFβ-1 release ($p > 0.05$). Results indicated that ScLL 5 μg/ml did not interfere in viability, and ScLL 2 μg/ml and 15d-PGJ$_2$ 1 μg/ml demonstrated reduced IL-6 release. Tested substances had no effect on TGFβ-1 release.

Keywords: Cell Survival; Fibroblasts; Lectins.

Introduction

The ideal procedure for avulsed teeth is immediate replantation, but this is not possible in most cases.[1] For this reason, the duration of the extra-alveolar dry time of the tooth, its storage medium, its replantation time and the survival of PDL cells are crucial factors determining the prognosis.[2] A more favorable prognosis after replantation must take these required factors into consideration. It is believed that storage media that can preserve PDL cell viability is more important than the extra-alveolar period.[2] However, under clinical conditions, it has been observed that patients do not know that the storage media is important, thus leading to delayed replantation.[3] In this situation, dry storage of avulsed teeth

causes death of PDL cells, and the presence of necrotic PDL remnants can trigger the development of root resorption.[1] The application of several therapies before replantation has been proposed to prevent or delay root resorption[4] in cases of late replantation, and thus increase the chance of avulsed tooth survival, including 2% acidulated phosphate fluoride on the root surface. However, this substance does not have active principles that could modulate immune inflammatory reactions triggering root resorption.[5] In this respect, a new and interesting challenge would be to investigate substances having the potential to modulate the inflammatory response, and act as a possible therapy to replace acid root treatments, in cases of late replanted teeth.

Some substances tested to treat the root surface before delayed tooth replantation have efficacy as osteoporotic therapeutic agents that inhibit osteoclast activity and modulate immune inflammatory reactions.[6,7,8] These substances include Emdogain, alendronate, odanacatib[6] and propolis.[7,8] In recent years, compounds of natural origin, obtained from plants such as curcumin and piperine, have been tested to formulate anti-inflammatory and anti-osteoclastogenic drugs having the potential to treat and prevent replacement resorption of replanted teeth.[9] In this respect, a recent study tested the effect of a plant lectin on stimulated gingival fibroblasts, with promising results[10] for future use as a possible therapy for delayed replanted teeth. Lectins are carbohydrate-binding proteins involved in various biological process, including cell-cell recognition, proliferation, migration, adhesion and biochemical responses.[11] The lectin isolated from the Euphorbiaceae *Synadenium carinatum* latex (ScLL) plant exhibits immunoregulatory properties.[12] This ability of ScLL to modulate inflammatory activity has been demonstrated in models of chronic inflammation, such as leishmaniose,[13] asthma[12] and neosporosis.[14]

Another component with immunomodulatory capability is 15-Deoxy-$^{\Delta 12,14}$-PG J_2 (15d-PGJ_2), a cyclopentenone-type PG that has demonstrated promising results in different experimental models *in vitro*[15] and *in vivo*.[15,16] 15d-PGJ_2 displays a wide spectrum of physiological activities, and is one of the terminal products of the cyclooxygenase (COX)-2 pathway.[16] It differs from other prostaglandins in several aspects, especially chemically and biologically, mainly due to its anti-tumor[15] and anti-inflammatory effects.[16] 15d-PGJ_2 was previously evaluated in a periodontitis mouse model, in which it exhibited immunomodulatory effects, by decreasing bone resorption and inflammatory responses.[16] However, more in-depth studies are of interest to the dental field, especially to evaluate the effect of 15d-PGJ_2 on other cells of the oral cavity, and compare this agent with other potentially immunomodulating substances.

The purpose of this study was to investigate the effect of 15d-PGJ_2 and ScLL on gingival fibroblasts stimulated with *Porphyromonas gingivalis* lipopolysaccharides (LPS) regarding viability and IL-6 and TGFβ-1 release. The hypotheses were that a) cells stimulated with LPS and treated with ScLL or 15d-PGJ_2 would demonstrate cell viability and TGFβ-1 release results similar to those of the positive control group; and b) IL-6 levels would be lower in the treated groups than in the negative control group.

Methodology

Cell culture

Immortalized human gingival fibroblasts (HFGs) (Cell Bank of Rio de Janeiro, Rio de Janeiro, RJ, Brazil) were cultured in T-50 culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) (Vitrocell Embriolife, Campinas, Brazil) and supplemented with 10% fetal bovine serum (FBS) (Vitrocell Embriolife), in a humidified incubator with 5% CO_2 and 95% O_2 at 37°C, until confluence. HFGs were trypsinized, and seeded in 96-well culture plates (Coastar Corp., Cambridge, USA) with 10% DMEM at a cell density of $2x10^4$ cells/well. After 24 h, the medium was gently removed from each well and replaced by *P. gingivalis* LPS 10 µg/ml (Invivogen, San Diego, USA) in 10% DMEM for 24 h. Then, the medium was removed and treated with ScLL (2 µg/ml, 5 µg/ml) (Herbarium of the Federal University of Uberlandia, UFU), and 15d-PGJ_2 (1 µg/ml, 2 µg/ml) (Sigma-Aldrich, St. Louis, USA) in 10% DMEM for 1 and 3 h. The positive control group was maintained in 10% DMEM (not LPS-stimulated), and the negative control group was stimulated with LPS. After the

incubation period, the cells were prepared for cell viability analysis and cytokine assay.

Cell viability assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay. After removing the medium, the cells were rinsed with a phosphate-buffer solution (PBS), and an MTT solution (5 mg/ml) was added to the cells, which were incubated at 37°C for 4 h to allow development of formazan crystals. The supernatant of each well was replaced with the same volume of dimethyl sulfoxide (Sigma-Aldrich) to dissolve the crystals. Optical density was measured at 570 nm using a microplate reader (Biochrom, Cambridge, UK, England). Cell viability evaluation was performed according to the absorbance level, and was expressed as a percentage of viable cells.

Cytokine assay

IL-6 and TGFβ-1 levels were analyzed after 24 h of cell exposure to ScLL and 15d-PGJ_2. Cytokine levels were measured in culture supernatants using a sandwich Elisa kit for IL-6 (PeproTech, Rocky Hill, USA) and TGFβ-1 (Sigma-Aldrich), according to the manufacturer's instructions. Absorbance was recorded using a microplate reader (Biochrom), at 405 nm wavelength for IL-6, and 450 nm for TGFβ-1. The results obtained for absorbance were interpolated into a standard curve, using Microplate Manager 4.0 software (Bio-rad, Hercules, USA).

Statistical analysis

MTT, IL-6 and TGFβ-1 data were subjected to analysis of normality using Shapiro-Wilk and Levene's tests. The MTT data were evaluated with log transformation followed by one-way analysis of variance (ANOVA) and Tukey's test to compare the experimental groups ($p < 0.05$). Data for IL-6 and TGFβ-1 were analyzed using one-way ANOVA followed by Tukey's test. Dunnett's test was performed to compare the control with the experimental groups. The statistical analyses were performed using SigmaPlot 12.0 software (Systat Software, San Jose, USA), with statistical significance set at $\alpha = 0.05$.

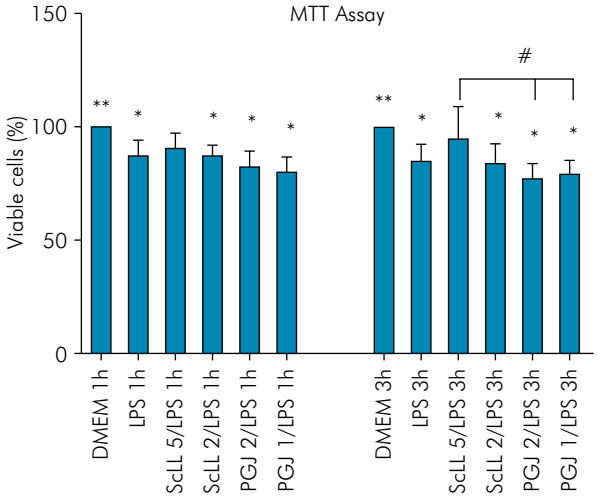
Results

Cell viability

The results for cell viability evaluated by MTT are shown in Figure 1. No significant difference was found among the experimental groups at 1 h ($p > 0.05$). However, ScLL 5 μg/ml presented higher cell viability than 15d-PGJ_2 1 μg/ml and 2 μg/ml, at 3 h ($p < 0.001$). The percentage of viable ScLL 5 μg/ml cells was similar to that of the positive control group (DMEM) in both periods evaluated ($p > 0.05$). All the other groups had lower levels of cell viability than the positive control, at 1 and 3 h ($p < 0.05$). The experimental groups were similar to the negative control (LPS) in both periods evaluated ($p > 0.05$), but not to the positive control ($p < 0.05$).

Release of IL-6 and TGFβ-1

The results for IL-6 and TGFβ-1 release are shown in Figures 2 and 3, respectively. The IL-6 level was statistically higher for the ScLL 5 μg/ml-treated and the 15d-PGJ_2 2 μg/ml-treated groups at 1 h, compared with the other treated groups and the positive control group ($p < 0.05$). Similar values were found for the negative control and the treated groups at 1 h, except



*$p < 0.05$ compared with positive control (DMEM). **$p < 0.05$ compared with negative control (LPS).

Figure 1. Effect of 15d-PGJ_2 and ScLL on cell viability of HGFs at 1 h and 3 h by MTT assay. Statistical significance was determined using one-way ANOVA and Dunnett's test. #$p<0.05$ compared among the experimental groups.

for 15d-PGJ$_2$ 1 µg/ml, which showed lower IL-6 release ($p = 0.04$). No significant differences were found among the groups at 3 h ($p > 0.05$), except for ScLL 2 µg/ml and 15d-PGJ$_2$ 1 µg/ml, which showed lower IL-6 release than the negative control group ($p < 0.05$).

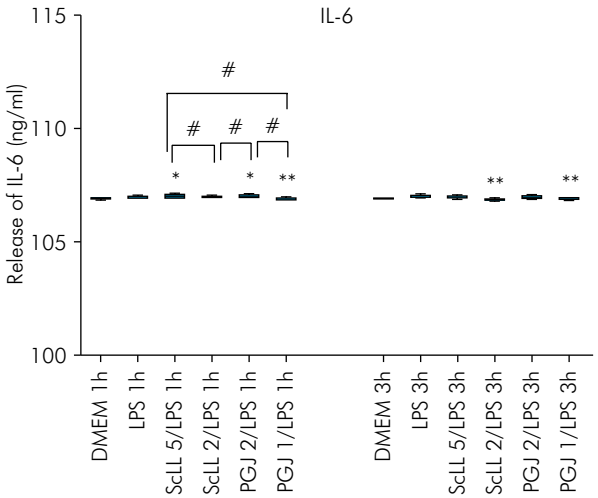
Regarding TGFβ-1 release, no significant difference was found among the groups in either evaluated period ($p > 0.05$).

Discussion

The first hypothesis was rejected, because cell viability analysis revealed lower levels of HFG viability in the treated groups than the positive control group, even though TGF-β release presented values similar to this group. The second hypothesis was accepted, because IL-6 release was lower in the treated groups than the negative control group. HFG is a immortalized cell line obtained from gingival tissue. It is important to note that the use of immortalized cell cultures performed in preliminary studies assessing drugs and medicines was recommended in the literature.[9,10] Moreover, established cell lines have enhanced reproducibility of results, an unlimited life span, and a higher rate of cell multiplication than primary culture cells.[17] There are many cell types in the oral environment, which may react differently to the test products, a possibility which should be carefully considered. Although the phenotypic and genotypic characteristics of gingival fibroblasts differ from those of periodontal fibroblasts,[18] immortalized PDL cells are not available for this protocol. This is why the HGF lineage was selected for this preliminary study.

In the present study, the cells were stimulated with LPS in order to mimic situations that occur clinically. Studies have shown that gingival fibroblasts express Toll-like receptors and CD14 in response to LPS.[17] It is known that LPS induces cell stress, associated with the activation of mitogen-activated protein kinase (MAPK), after an inflammatory response.[19] MAPK mediates the intracellular signaling related to cellular activities, such as apoptosis, cell differentiation and cell survival.[20] Studies using fibroblasts activated with LPS in drug tests are recommended in this area.[10]

Considering that LPS could interfere in cell viability, the current research evaluated its presence by MTT assay. Tetrazolium salts have become widely used in cell biology assays, because of their ability to convert yellow MTT tetrazolium salt into dark blue formazan crystals, by the mitochondrial activity of living cells. Compared with other viability assays,



*$p < 0.05$ compared with positive control (DMEM). **$p < 0.05$ compared with negative control (LPS).

Figure 2. Release of IL-6 in HGFs examined by Elisa at 1 and 3 h. Statistical significance was determined using one-way ANOVA and Dunnett's test. #$p<0.05$ compared among the experimental groups.

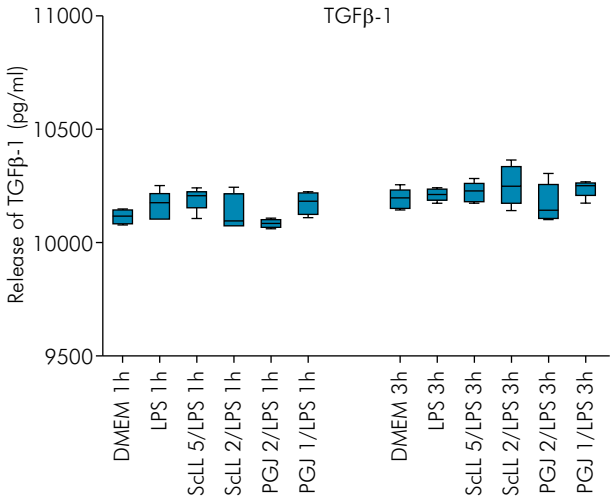


Figure 3. Release of TGFβ-1 in HGFs examined by Elisa at 1 and 3 h. Statistical significance was determined using one-way ANOVA and Dunnett's test.

the MTT assay is simple, fast, precise, sensitive and reproducible.[21] In the current study, the highest viability levels were found for the positive control group. This result can be attributed to the absence of LPS, which may trigger the cell death mechanism.[22] The fact that ScLL 5 μg/ml showed viability similar to the positive control group suggests that this ScLL concentration may act at any point of the apoptotic pathway, blocking the molecular events in the apoptotic process, thus maintaining viability levels similar to basal cell parameters. However, it should be emphasized that, in the present study, the LPS percentage remained above 80%, despite its reduced cell viability.

Analyses of IL-6 and TGFβ-1 release were performed based on the belief that LPS could induce fibroblasts to display some sort of pro-inflammatory reaction, and that treatment with ScLL and 15d-PGJ_2 could have a regulatory effect on this parameter. IL-6 is produced by human fibroblasts when stimulated by bacteria and their products, and plays a key role in regulating the immune response.[23] Furthermore, IL-6 is involved in a broad spectrum of biological activities, plays a role in cellular defense, and acts on several target cells. When this cytokine acts on bone, it affects mainly osteoclastogenesis and bone resorption.[23] Taking into consideration the anti-inflammatory role of the tested substances, it was expected that lower levels of IL-6 would be found in the treated groups than in the negative control group, in the current study. However, only ScLL 2 μg/ml and the 15d-PGJ_2 1 μg/ml showed a significant reduction in IL-6 release. This result may indicate that these substances were able to modulate the release of this inflammatory cytokine in stimulated fibroblasts, in a concentration-dependent manner.

Another relevant factor evaluated was release of the TGFβ cytokine, which plays an important role in promoting fibroblast differentiation at inflammation and repair sites.[24] Curiously, no differences were found in the TGFβ-1 levels in the different groups of the current study. Possibly, the 24 h time period established for detection of TGFβ-1 levels in the present study was not enough to release this cytokine in gingival fibroblasts. Furthermore, the amount of LPS used to stimulate HGF cells may not have been enough to induce TGFβ-1 release during this time. It has been demonstrated that TGF-β production in PDL cells versus gingival fibroblasts differs, and that this difference could be attributed to the highest concentration of *P. gingivalis* LPS used to stimulate this production.[25] Although it was not evaluated in the present study, joint production of TGFβ-1 and IL-6 may induce release of the Th17 cell profile,[26] which can result in tissue inflammation, since they have been found to occur in chronic periodontal inflammation.[27] Regarding the beneficial results of ScLL, this study was unable to define the effect of this substance on prolonged extra-alveolar periods, since only the periods of 1 and 3 h were evaluated. Therefore, more studies evaluating other cytokines, other cellular profiles and different experimental periods should be performed to further advance the present findings.

Despite the limitations of the present study, the results presented here suggest that lectin ScLL and 15d-PGJ_2 may be promising in dentistry, specifically in lower concentrations. This reflects their positive effect on the parameters evaluated, such as cell viability and IL-6 and TGFβ-1 release. However, it should borne in mind that 15d-PGJ_2 is a commercial product sold in small aliquots, thus representing a higher cost and greater difficulty in handling, in comparison with ScLL. This is why preference should be given to ScLL in future research to investigate biological models of greater complexity.

It is important emphasize that ScLL is a protein with significant immunoregulatory activity, like that of leukocyte trafficking and inhibition of TH2 cytokine production;[12] however, it has no nutritional potential. Thus, the main objective of this lectin is to control immune inflammatory reactions, which trigger root resorption. Application of this substance as a product for avulsed teeth should be associated with a storage media that has the nutritional ability to combine its modulatory property with maintenance of cell viability.

Therefore, more research using different *in vitro* and *in vivo* models is required to evaluate the potential of ScLL as an immunomodulator and anti-resorption agent for avulsion therapy. Moreover, future research

should evaluate the use of immunomodulator agents together with storage media of nutritional capability for use as a product for avulsed teeth.

Conclusion

Based on the results of the present study, it can be concluded that ScLL 5 µg/ml did not interfere in cell viability. In relation to the parameter of IL-6 release, ScLL 2 µg/ml and 15d-PGJ$_2$ 1 µg/ml demonstrated reduced levels of this inflammatory cytokine. None of the concentrations tested had any effect on TGFβ-1 release.

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