Grape Seed Extract Down-modulates Oxidative and Inflammatory Responses of Oral Mucosa Cells

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Abstract

Background: Proanthocyanidins (PAs) are bioflavonoids extracted from seeds and fruits that act as cross-linkers in collagen molecules and act as matrix metalloproteinase (MMP) regulators. Therefore, the use of these compounds seems to be a potential adjunctive therapy for traditional periodontal treatment. This study investigated the potential of PAs extracted from grape seeds (grape seed proanthocyanidins extract – GSPE) to modulate the oxidative and inflammatory response of oral mucosa cells.

Materials and methods: Non-cytotoxic concentrations of GSPE were selected for oral keratinocytes and gingival fibroblasts. Cells were seeded in 96-well plates and exposed to tumor necrosis factor-alpha (TNF-α) (100 ng/mL) and then treated with GSPE (0.001%) in serum-free culture medium. After 24-h-incubation, oxidative cells response (synthesis of nitric oxide), synthesis of interleukin-6, MMP-2, MMP-3, and MMP-9 was measured by ELISA immunoassay, as well as gelatinolytic activity (zymography). Data were analyzed by analysis of variance and Tukey tests (a = 0.05).

Results: TNF-α-challenged cells exhibited the highest rates of NO and inflammatory mediators' synthesis. Reduction of NO synthesis, downregulation of interleukin-6, MMP-2, and -9, as well as decrease in the gelatinolytic activity of fibroblasts was observed in GSPE groups.

Conclusions: GSPE is capable of modulating the oxidative and inflammatory responses of oral mucosa cells.

Keywords: Periodontal disease; matrix metalloproteinases; proanthocyanidins; fibroblasts; keratinocytes

Introduction

Periodontal diseases are chronic inflammatory conditions where, at the initial stages, only oral mucosa is involved, characterized by exacerbated inflammatory and oxidative responses and increased pro-inflammatory cytokine expression and other inflammatory mediators, such as nitric oxide. The development and severity of these diseases depends on the interaction between pathogenic microorganisms and local immunological host response (Akalin *et al.,* 2005; Franco *et al.,* 2017).

It has been demonstrated that periodontal diseases are regulated by a positive feedback with inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Franco *et al.,* 2017). Besides the efect of these inflammatory mediators on the severity of periodontal disease (Franco *et al.,* 2017), these molecules also enhance the synthesis and local activity of metalloproteinases and other collagenolytic enzymes, accelerating the disorganization of extracellular matrix (Amar *et al.,* 2017).

Matrix metalloproteinases (MMPs) are proteolytic enzymes that enable the cleavage of collagen fibrils and extracellular molecules. These enzymes play a very important role in the formation and remodeling of epidermal and mesenchymal tissues (Apte *et al.,* 2015). However, the overexpression of MMP-2 and MMP-9 by fibroblasts, osteoblasts, osteoclasts, and inflammatory cells, triggered by mediators of inflammation and by microorganisms products, accelerates local soft tissue disorganization and mineral resorption (Birkedal-

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Hansen, 1993; Feghali *et al.,* 2012; Franco *et al.,* 2017). Besides regulating extracellular matrix components, MMPs also participate in inflammatory pathways. These enzymes are up-regulated by pro-inflammatory cytokines and act by inflammatory mediators themselves, creating a positive feedback on local inflammatory reaction (Epasinghe *et al.,* 2013; Cui *et al.*, 2017).

In the oral cavity, these enzymes are mainly expressed by gingival fibroblasts which are major contributors for the synthesis and homeostasis of the connective tissue involved in periodontal inflammation. The importance of regulation of MMPs on periodontal diseases has already been demonstrated. The expression, synthesis and activity of these enzymes are directly related to the severity of periodontal diseases, as a positive relationship is observed for MMPs and destruction of periodontal tissues (Ejeil *et al.*, 2003).

According to previous reports, conventional periodontal therapy alone, which is able to locally reduce the MMPs synthesis, shows limited results on this biological event (Gonçalves *et al.,* 2013). Therefore, modulation of the gingival inflammatory and oxidative responses could stimulate tissue healing, accelerating the resolution of periodontal diseases (Birkedal-Hansen, 1993; Franco *et al.,* 2017).

Previous efforts have been made to evaluate the role of bioflavonoids on periodontal diseases control (Lombardo *et al.,* 2015; Boelen *et al.,* 2019; Bunte *et al.,* 2019; Golub and Lee, 2020). While recent investigations have focused on the effects of these biocomponents on the regulation of MMPs, *in vitro* studies have demonstrated that these molecules regulate the oxidative response of gingival fibroblasts (Bodet *et al.,* 2007; Lombardo *et al.,* 2015; Franco *et al.,* 2017). Grape seed proanthocyanidins (GSP) are bioflavonoids capable of regulating the inflammatory and oxidative responses of different cell types, such as osteoblasts (Bagchi *et al.,* 2000). Besides the antimicrobial, immunomodulatory and anticarcinogenic properties, these molecules can also modulate the expression and activity of MMPs (Lee *et al.,* 2007; Lee *et al.,* 2008; Govindaraj *et al.,* 2011; Bladé *et al.,* 2016; Richa *et al.,* 2017).

Therefore we have proposed that use of GSPs could be a suitable therapeutic and adjunctive treatment for periodontal diseases. To evaluate this hypothesis, this study assessed the potential of proanthocyanidins extracted from grape seeds (GSPE) on the modulation of oxidative and inflammatory response of oral mucosa cells.

Materials and methods

Cell culture

Oral keratinocytes and gingival fibroblasts were selected for this investigation. Oral keratinocytes (OK) (ATCC-PCS 200-014), were maintained in complete cell culture medium (DMEM, Gibco, Carlsbad, CA, USA) containing 1% of antibiotic and antimycotic solution (10.000 units/mL penicillin, 10.000 µg/mL streptomycin, 25 µg/mL Fungizone® - Gibco) and 10% fetal bovine serum (FBS). Gingival fibroblasts (HGF) were obtained from a non-smoker, systemically healthy young individual that was not under continuous medication. The gingival fragment was surgically collected from a periodontally healthy site during an impacted third molar extraction following procedure previously approved by the Ethics Committee (CAAE: 74823317.0.0000.5416). Tissue fragment was immediately placed in a sterile tube containing cell culture medium (DMEM), supplemented with 10% of FBS and 1% of antibiotic and antimycotic solution. Cells were isolated by enzymatic digestion using collagenase type I (Worthington Biochemical Corp, Lakewood, NJ, USA) in FBS-free DMEM (3 mg/mL) for 24 h in a humidified incubator (37°C and 5% CO_2). After this period, disaggregated cells were transferred to a cell culture flask containing complete DMEM. Both cell lines were subcultured using 25% trypsin (Gibco).

Grape seed extract

Grape seed proanthocyanidins extract (GSPE) was obtained from Shaanxi Sinuote Biotech Co. Ltd, (Shaanxi, CH). It corresponds to a powder grape seed extract from the Vitis vinifera species, which is rich in proanthocyanidins (95% proanthocyanidins, lot #CPTZ20130512).

Analysis of cell viability

A non-cytotoxic concentration of grape seed proanthocyanidins extract was selected after assessing the viability of OK and HGF exposed for 24 h to different concentrations of GSPE. Cells were seeded in 96-well plates in complete DMEM and incubated for 24 h. Then, the culture medium was replaced with fresh FBS-free DMEM containing different concentrations of grape seed proanthocyanidins extract (GSPE): 0 (control group); 0.01%; 0.005%; 0.001% and 0.0005%. Cells in the positive control group were maintained in FBS-free DMEM, while the negative control group, where the lowest cell viability was expected, was exposed to H_2O_2 at 3%. After exposing the cells to the GSPE for 24 h, the viability of these cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay. For this purpose, the GSPE was aspirated and the MTT salt (Sigma-Aldrich, St Louis, Mo, USA) (5 mg/mL in phosphate buffered saline solution-PBS) diluted in FBS-free DMEM (1:10) was applied to the cultured cells. After 4 h incubation at 37°C, the formazan crystals resulting from the cleavage of MTT salt were dissolved in acidified isopropyl ethanol and the purple solutions were evaluated by spectrophotometry (570 nm) (Synergy H1 microplate reader, BioTek

Instruments, Winooski, VT, USA). Cell viability rate for the GSPE groups was determined according to the control group (100% cell viability).

Experimental design

OK and HGF were seeded in wells of 96-well plates (10^4 cells/well) in complete DMEM for 24 h. Then, cells were challenged with an inflammatory stimulus (TNF-α, 100 ng/mL) for 24 h and treated or not treated with the grape seed proanthocyanidins extract (GSPE) at 0.001% for an additional 24 h. Cells exposed solely to TNF-α or not treated (maintained in FBS-free DMEM) were used as positive and negative controls, respectively.

Oxidative cell response – Nitric Oxide synthesis

The oxidative response of OK and HGF exposed to TNF-α and/or GSPE was assessed by the quantification of nitric oxide (NO), which is a potent oxidative and inflammatory mediator widely used to determine the cell oxidative response (Kendall *et al.,* 2001), produced by the cultured cells (n = 6). This protocol is based on the accumulation of NO in cell culture medium, and measured using the diazotation of Griess reagent (Sigma-Aldrich, St Louis, MO, USA). After treatment, 50 mL- aliquots of each sample were transferred to wells of a 96-well plate, and then incubated with 50 mL of Griess reagent for 10 min in a dark environment. Then, absorbance was determined in spectrophotometer at 540 nm (Synergy H1).

Synthesis of IL-6 and MMP-2 -3 and-9

Synthesis of the pro-inflammatory cytokine interleukin-6 (IL-6) and matrix metalloproteinases-2, -3, and -9 were evaluated by enzyme-linked immunoassay (ELISA), following the manufacturer's instructions (Duo Set Kit, R&D Systems, Minneapolis MN, EUA) (n = 6).

Gelatinolytic activity

The gelatinolytic activity of OK and HGF was determined by *in situ* zymography, which was based on degradation of fluorescein-conjugated gelatin (EnzCheck® gelatinase/collagenase Assay Kit, Molecular Probes, Eugene, OR, USA). Cells exposed or not exposed to TNF-α (100ng/mL) and/or GSPE were covered with 200 mL of the gelatin and incubated at 37°C and 5% CO_2 for 1 h. The gelatin degradation, regarded as positive degradation/gelatinolytic activity, was detected under fluorescence microscope (qualitative data; n = 2) and fluorimeter (quantitative data; n = 6) (498 nm/530 nm; Synergy H1).

Results

Cell viability

OK and HGF presented similar responses to GSPE. Cells exposed to H_2O_2 showed the lower rates of viability. Cells treated with GSPE at 0.01% and 0.005% demonstrated diminished viability after 24 h of contact, while this extract at 0.001% and 0.0005% showed mean cell viability similar to control group (Figure 1a & b).

Synthesis of Nitric Oxide

TNF-α-challenged OK and HGF presented increased oxidative response. GSPE-treated cells showed oxidative response similar to negative control group (FBS-DMEM) for both cell lines. Associated to TNF-α, a down-modulation of NO was observed and this modulation was more evident for HGF (Tukey, $p > 0.05$; Figure 2 a & b).

Synthesis of IL-6, MMPs and Gelatinolytic activity

In comparison to other groups, the exposure of cells to the inflammatory stimulus increased the IL-6 significantly (Figure 3 a & b) as well as MMP-2, -3, and

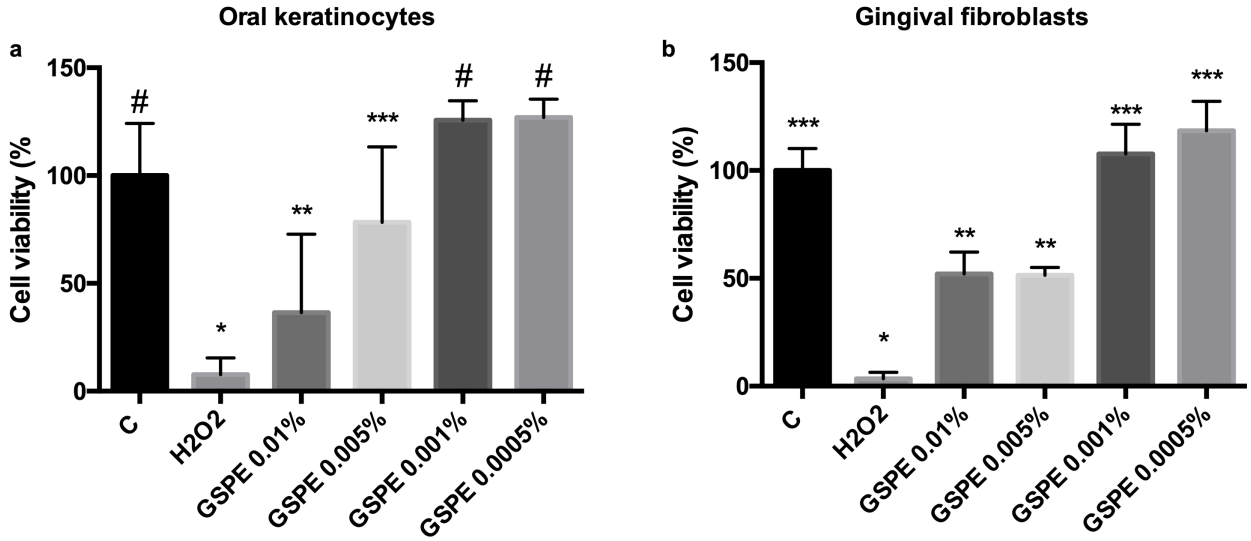

Figure 1. Viability of oral keratinocytes (a) and gingival fibroblasts (b) exposed to grape seed proanthocyanidins extract at different concentrations.

-9 synthesis ($p < 0.05$) (Figure 4 a, b & c). The application of GSPE in HGF previously exposed to TNF-α downregulated the synthesis of IL-6 and MMP-2 and -9. However, synthesis of MMP-3 by OK was not regulated by GSPE.

The analysis of gelatin degradation demonstrated that OK and HGF submitted to inflammatory stimulus had the highest activity, which was significant in comparison to other groups ($p < 0.05$), and it corresponds to cells collagenolytic and gelatinolytic activities. In the other hand, HGF exposed or not to TNF-α and then treated with GSPE exhibited a significant decrease in the matrix-degradation in comparison to both positive and negative control groups (Tukey, $p < 0.05$) (Figure 5b). However, this downmodulation was not observed for OK (Tukey, $p > 0.05$) (Figure 5a).

Discussion

Gingival fibroblasts are responsible for the maintenance of homeostasis of the periodontium, mainly by secreting collagen and other extracellular matrix proteins. These cells also express inflammatory mediators, such as inflammatory cytokines and chemokines, and show a strong responsiveness to pro-inflammatory cytokines and microorganisms' products (Bartold *et al.,* 2000). Due to the importance of these cells on the initiation and severity of periodontal disease, the great majority of studies investigating cellular and molecular events associated with periodontal disease are designed using solely gingival fibroblasts. Moreover, the secretion and activity of MMP-2 and -9 by gingival fibroblasts and the role of these enzymes on the severity of periodontal diseases has previously been reported (Nazar Majeed *et al.,* 2016). In association to the inflammation and disorganization of connective tissue (Bartold *et al.,* 2000; Bosshardt and Lang, 2005), junctional epithelium, which resembles the epithelial tissue of gingiva, also has important functions for periodontal protection and homeostasis and also participates in the progression of periodontal diseases. Therefore, this investigation also

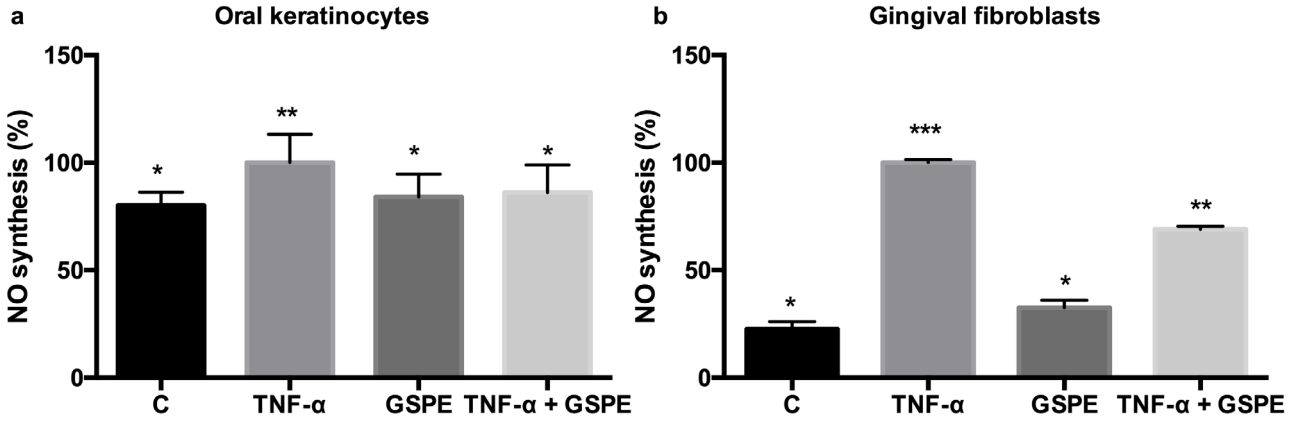

Figure 2. Nitric Oxide production by oral keratinocytes (a) and gingival fibroblasts (b) exposed to grape seed proanthocyanidins extract for 24 h, associated or not with a previous positive inflammatory stimulus tumor necrosis factor-alpha. Control (C) was no treatment Groups identified by different symbols indicate statistical difference. (Tukey, $p < 0.05$).

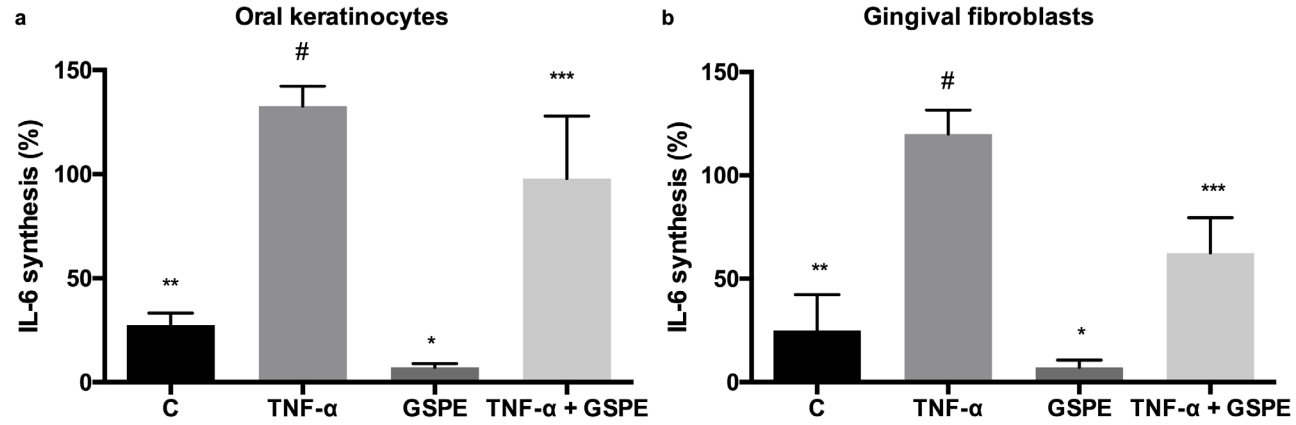

Figure 3. Synthesis of IL-6 by oral keratinocytes (a) and gingival fibroblasts (b) exposed to grape seed proanthocyanidins extract (GSPE) for 24 h, with or without previous stimulus with tumor necrosis factor-alpha. Control (C) was no treatment Groups identified by different symbols indicate statistical difference. (Tukey, $p < 0.05$).

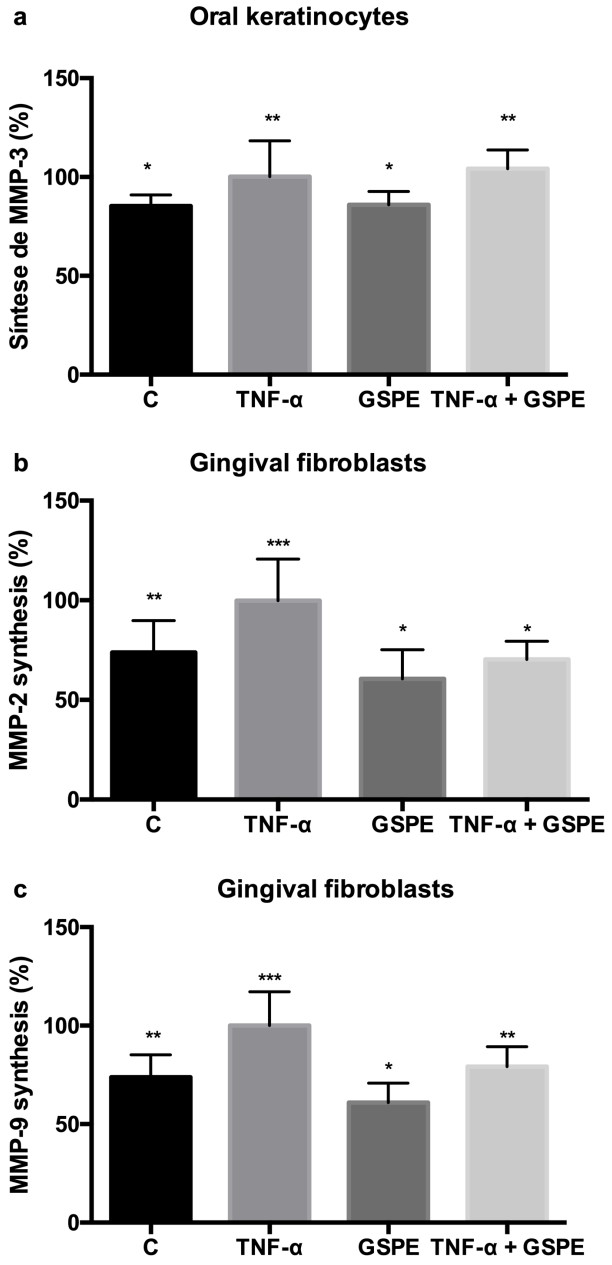

Figure 4. Synthesis of matrix metalloproteinase-3 (MMP-3) by oral keratinocytes (a) and MMP-2 (b) and -9 by gingival fibroblasts (c) exposed to grape seed proanthocyanidins extract (GSPE)for 24 h, associated or not with a previous positive inflammatory stimulus tumor necrosis factor-alpha. Control (C) was no treatment. Groups identified by different symbols indicate statistical difference. (Tukey, $p < 0.05$).

included the evaluation of epithelial tissue, by assessing the effects of GSPE on oral keratinocytes.

At first, it was important to select GSPE concentrations that did not result in cytotoxic effect on oral mucosa cells. Despite there being several other investigations that have evaluated the effects of bioflavonoids on fibroblasts and other mesenchymal cells, only a few studies have used gingival fibroblasts to determine the potential effects of these molecules (Bodet *et al.,* 2007; Tipton *et al.,* 2017). After identifying four distinct concentrations (0.0005%–0.01%), an intermediate concentration was selected (0.001%) based on the major aim of this study, which was to control oxidative and inflammatory response. When evaluating the cytotoxicity of anthocyanins, researchers have reported results similar to those found in the present study, where toxic effects were observed for concentrations over 0.001% (Bodet *et al.,* 2007; Tipton *et al.,* 2017). Regarding oral keratinocytes, the literature still lacks the evaluation of bioflavonoids on this type of cell.

The oxidative response, characterized by synthesis of oxygen species such as nitric oxide, has been considered as an indicator of tissue toxicity and inflammatory reaction; in periodontal diseases, specifically, oxidative stress seems to play a central role in the synthesis of pro-inflammatory mediators (Wang *et al.,* 2017). This investigation demonstrated that gingival fibroblasts and oral keratinocytes exposed to GSPE exhibited a significantly lower oxidative response than non-treated cells. Similarly, the potential of GSPE on reducing the oxidative response of mesenchymal cells has already been demonstrated by other researchers (Shao *et al.,* 2006; Zhang *et al.,* 2014), who also found a correlation between the cytotoxicity and survival rate of these cells and the increased and decreased oxidative stress, respectively.

IL-6 is a pro-inflammatory cytokine strongly associated with the severity of periodontal diseases; increased levels of this cytokine induce activation of macrophages and indirectly influence bone destruction (Naruishi and Nagata, 2018). Moreover, as observed for TNF-α, IL-6 has been described as a potent regulator of MMPs (Franco *et al.,* 2017), and exacerbates the synthesis of other inflammatory mediators, such as TNF-α and IL-6, creating a positive feedback effect on the local inflammatory reaction and exacerbating the imbalance of extracellular matrix. In this study, increased levels of IL-6 were detected for both cell lines after challenge with TNF-α, confirming the potential role of epithelial tissue and the underlying connective tissue on the regulation of periodontium homeostasis and disease.

These inflammatory mediators (IL-6 and MMPs) were downregulated following the application of GSPE which was also associated to the decreased synthesis of oxidative products, demonstrating that GSPE may regulate periodontal inflammation downstream and control tissue degradation processes. An exception was observed for MMP-3 synthesis by oral keratinocytes, after TNF-α challenge. This may be due to some peculiarity of this enzyme such as the fact that MMP-3 can be activated even in the absence of zinc and also shows a transmembrane domain, which can hamper the regulation of this enzyme (Cui *et al.,* 2017).

These data are consistent with the literature as well, which demonstrate that distinct sources of proanthocyanidins extracts also down-regulate the synthesis of pro-inflammatory cytokines and MMPs. It has also been suggested that these bioflavonoids act by the inactivation

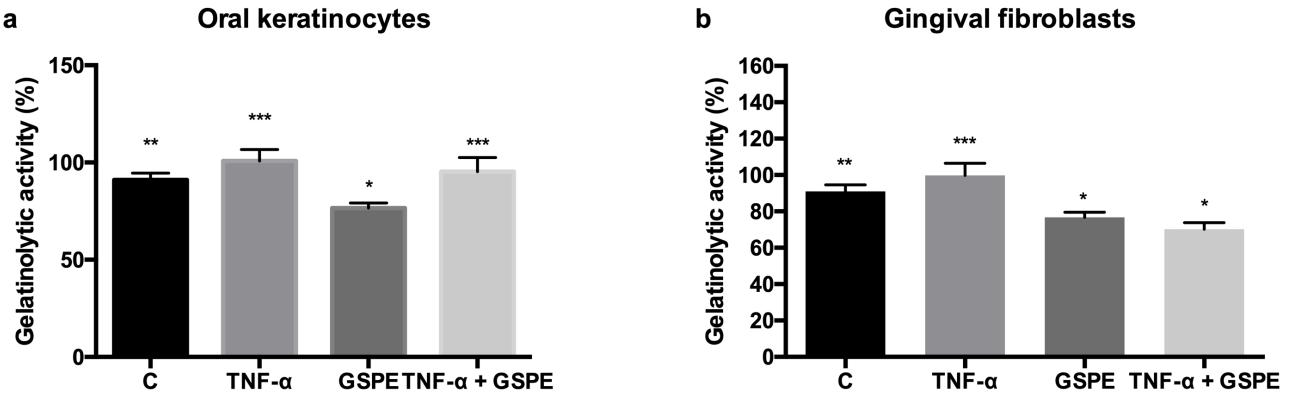

Figure 5. Gelatinolytic activity of oral keratinocytes (a) and gingival fibroblasts (b) exposed to tumor necrosis factor-alpha (100ng/mL) and then treated with grape seed proanthocyanidins extract (GSPE). Control (C) was no treatment. Groups identified by different symbols indicate statistical difference. (Tukey, $p < 0.05$).

of the transcription factor activator protein-1 (AP-1), or by inhibiting Nf-kB p65, which may result in decreased MMP production (Bodet *et al.*, 2006; La *et al.*, 2010; Feghali *et al.*, 2012).

The progression and severity of periodontal diseases are directly related to increased concentrations of pro-inflammatory cytokines, such as TNF-α and IL-6 (Kendall *et al.,* 2011), playing an important role in the wound healing process of oral mucosa by regulating the balance of synthesis and degradation of collagen and other extracellular matrix proteins, further resulting in the maintenance of tissue homeostasis (Nazar Majeed *et al.,* 2016). However, other biomarkers have also been considered for the diagnosis and control of periodontal inflammation, such as matrix metalloproteinases (MMPs).

These enzymes can be considered as potential diagnostic biomarkers, because, as inflamed, periodontal tissues show exacerbated synthesis and activity of these enzymes, which results in unbalanced degradation of connective tissue as well as indirect stimulation of bone resorption (Feghali *et al.,* 2012). This stimulation can be triggered by microorganisms and its toxic products, such as lipopolysaccharides (LPS), as well as by a positive feedback related to inflammatory mediators, such as cytokines and oxidative products (Kobayashi *et al.,* 2003; Kothari *et al.,* 2014).

This investigation has demonstrated the safety of the application of a grape seed extract containing proanthocyanidins on gingival fibroblasts and oral keratinocytes and also the effectiveness of this extract on regulating inflammatory and oxidative response by these cells. However, it is important to consider the limitations of the present investigation, since it is a pre-clinical *in vitro* investigation that was conducted in monolayer cell culture model and the use of a natural bioflavonoids that can show variations of activity according to culture conditions, cell seeding and extraction. Using these data, the suitability of this therapeutic protocol may be further evaluated to propose future clinical protocols.

The modulation of MMPs has already proven to be a suitable and effective strategy to control different inflammatory conditions, specially by solutions containing MMP-inhibitors (Feghali *et al.*, 2012). Considering periodontal disease is a chronic inflammatory condition, the results of the present study can be regarded as interesting to establish further methods to control the progression of such oral conditions.

Acknowledgments

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