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Anti-Inflammatory and Antibacterial Activity of Vitamin D in Human Gingival Fibroblasts and Periodontal Ligament Cells

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SUMMARY

It has been recently demonstrated that Vitamin D can exert a modulatory role on the inflammatory and immune responses by acting on several cells and tissues. The aim of our study was to investigate the *in vitro* effects of vitamin D3(VD) on antibacterial activity against *Porphyromonas gingivalis (Pg) and Streptococcus pyogenes (GSA) and to evaluate the* expression of pro-inflammatory (IL-8) and anti-inflammatory (IL-10) cytokines in Human Gingival Fibroblasts (hGF) and Periodontal Ligament Cells (hPDLc).

Primary hGF and hPDLc pre-treated or not by VD (10^{-8}mol/L) were exposed to Pg and GSA for 24h. Cell viability by MTT-test and production of IL-8 and IL-10 were evaluated. Bacterial growth by CFU counting was also assessed.

When infected by Pg and GAS, VD treatment hugely increased the viability in HGF (50.2% to 89.1%, 47.9% to 86.6%) and HPLc (60.8% to 94.8% and 59.5% to 85.9%). Treatment with VD decreased bacterial growth by approximately 30%.

In VD-treated HGF and HPLC, IL-8 concentration decreased to 31.4% and 44.6% for Pg and 30.8% and 28.0% for GSA. IL-10 increase was 188.9% and 221.8% for Pg and 220.8% and 247.9% for GSA.

The obtained results support the hypothesis of a protecting role of VD on periodontal cells exposed to bacterial infection, increasing cell viability, reducing their inflammatory response, and exerting an antibacterial effect.

INTRODUCTION

Periodontitis is an inflammatory disease associated with a bacterial infection that leads to the destruction of the periodontium. As far as we know today, an infective biofilm is necessary in periodontitis initiation, however the inflammatory response of the periodontal tissues to infection is influenced by environmental factors as well as by genetic factors. The observation that periodontitis is a complex disease entity with a multifactorial etiology has led to the search for risk factors that ultimately could modify cellular and subcellular mechanisms of the host response influencing the pathogenesis of the disease.[1,2]

Diabetes mellitus, smoking, poor oral hygiene, acquired or congenital immunodeficiencies, systemic diseases, stress, which - alone or in combination - can contribute to unfavourable tipping of the homeostatic balance, increase susceptibility to periodontitis.[3] In this respect, nutrition may be of great importance since it has been implicated in a number of inflammatory diseases and conditions.[4]

The better understood function of vitamin D is to support calcium homeostasis. Vitamin D regulates serum calcium and phosphate in bone by increasing intestinal calcium absorption and via PTH decreasing the release of calcium from bone and stimulating reabsorption of calcium in the renal distal tubule.[5,6]

However, the presence of vitamin D receptors (VDR) in many tissues not involved in skeletal health, suggested that vitamin D could have additional effects.[7]

It is through this binding of vitamin D that it is ultimately able to exert its effect on the cells of the immune system. All the cells of the immune system, have been shown to express VDR and are able to convert vitaminD to its active form.[8] Moreover, vitamin D has a role as a potent stimulator of antimicrobial peptides in innate immunity. The production of cathelicidin and some defensins (defensinshBD-2) in the human body is dependent on sufficient circulating 25(OH)D.[9]

Antimicrobial peptides may have antibacterial and LPS neutralizing activity against periodontapathogens.[10]

In contrast to its stimulatory role in innate immunity, vitamin D inhibits adaptive immunity, thus reducing the inflammatory response arising from cytokine and immunoglobulin production.[11,12]

Vitamin D may prevent an over reaction of the inflammatory response in the adaptive immune system preventing further cell or tissue damage by inflammation. In summary, all these data support the idea that the presence of vitamin D results in a more tolerogenic immune system .[5]

Porphyromonas gingivalis, a Gram-negative anaerobic black-pigmented rod, has been implicated as a crucial etiologic agent in the initiation and progression of periodontitis P. gingivalis requires a preformed streptococcal substratum for its incorporation into a biofilm.[13]

This microorganism produces several virulence factors that stimulate Human periodontal ligament cells (hPDLc) to produce inflammatory mediators, such as prostaglandin E2, matrix metalloproteinases (MMPs) and proinflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-12 and TNF-α. These mediators further activate the periodontal cells to mount excessive host inflammatory responses, resulting in disease progression and periodontal destruction.[14]

Streptococcus pyogenes (also defined group A streptococci [GAS]) is a common colonizer of the mucosal layers in the mouth, nose, and pharynx, but it is also ubiquitous and of considerable public health importance. The streptococci are part of the endogenous microbial flora. Disease may result from circumvention of the normal specific or nonspecific host defense mechanisms; GAS have been shown to enter and survive intracellularly in macrophages,[15] neutrophils,[16,17] , and epithelial cells[18,19] and shares with *P. gingivalis* the production of a cysteine proteinase that have been implicated in periodontal pathogenicity.[20] One shared feature of the two pathogens is the ability to either inhibit or evade innate host responses. This observation led to the speculation that the strong association of these bacteria with diseased sites may be related to their ability to disrupt oral innate defense functions facilitating untoward host interactions with the microbiota residing in the mucosal layer.[1]

Human periodontal ligament cells (hPDLc), mainly consisting of fibroblasts from periodontal ligament tissues, have been proven to take part in not only the maintenance of periodontal tissues but also the regulations of periodontal inflammation. *P. gingivalis* and its components can enhance the production of proinflammatory cytokines in hPDLc, In a previous study, it was found that hPDLc express vitamin D receptor (VDR).[21]

Human Gingival Fibroblasts (hGF) are a key cellular component of gingival connective tissue and are highly involved in tissue reactions to the first microbiological challenges that develop in the gingival sulcus, being involved both in signaling, responding and reparative functions.[22] VDR was demonstrated to be active also on hGF.[23]

However, it is unclear that whether vitamin D may regulate the expressions of pro-inflammatory and anti-inflammatory cytokines in hPDLc and hGF so as to regulate the pathogenic pathway of periodontitis.

AIM: The aim of current study was to examine *in vitro* the effects of exogenous active form of vitamin D3 ($1\alpha,25[OH]_2D_3$) on antibacterial activity against *P. gingivalis and S. pyogenes*; in addition we investigated the effect of the vitamin D on the inflammatory immune response of cultured hGF and hPDLc with respect to expression of a proinflammatory cytokine (IL-8) and an anti-inflammatory cytokine (IL-10).

MATERIALS AND METHODS

Reagents and bacterial strains - Vitamin D3 (1α,25[OH]$_2$D$_3$) was obtained from Elifab (Elifab s.r.l. Napoli, Italy). Vitamin D3 (VD) was dissolved in 0.1% of absolute ethyl alcohol at a concentration of 10^{-10} mol/l, 10^{-9} mol/l and 10^{-8} mol/l.

P. gingivalis from the American Type Culture Collection (ATCC 33277) was anaerobically grown at 37 °C for 2 to 3 days in Trypticase soy (TS) broth (30 g/l) containing 1 g/l yeast extract (Difco), 1 g/l glucose, 0.5 g/l potassium nitrate, 1 ml/l sodium lactate (Sigma L-1375), 0.5 g/l sodium succinate and 1 g/l sodium fumerate; after autoclaving, filter-sterilized supplements were added (0.4 g/l sodium carbonate; 0.005 g/l hemin [σ H-2250]; 0.4 g/l cysteine; and 0.001 g/l vitamin K [σ M-5625]). Late logarithmic-phase cells were employed; bacteria were washed twice in sterile PBS.

S. pyogenes was isolated from the sample from a patient with pharyngotonsillitis. *S. pyogenes* cultures were grown overnight at 37°C in fresh sterile Muller-Hinton broth (Becton-Dickinson Difco) and after incubation, bacteria were washed twice in sterile PBS.

Following the final resuspension, *P. gingivalis* and *S. pyogenes* were diluted to an OD600 nm in sterile PBS, which corresponded approximately to10^7CFU/ml, to obtain the MOI of 100. From this, stock bacteria were diluted 10^6 to obtain the MOI of 50.

Cell culture - A pool of periodontally healthy premolars and molars were obtained from young individuals (aged 22 to 36 years) starting orthodontic extractive treatments after informed consent.

Teeth were washed twice in sterile PBS supplemented with antibiotics (100 U/ml penicillin, 125 ug/ml amphotericin B).

Gingival tissue was isolated at the cemento-enamel junction of the extracted teeth by means of a surgical blade. The harvested tissue was rinsed several times in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Milan, Italy) containing antibiotics (penicillin 100 U/ml; streptomycin 125µg/ml and amphotericin B 5µg/ml). The tissue was cut into small pieces and cultured with a medium containing 10% fetal bovine serum (FBS; Gibco Invitrogen), L-glutamine (600µg/ml), penicillin (100 U/ml), and streptomycin (125µg/ml) in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The cells that grew from the explant tissues were subcultured. Cell cultures used in all experiments were between passages 2 and 4.[14]

Primary hPDLc were scraped with a sterile surgical scalpel from the middle part of the roots and put in a tube with 1 ml of PBS containing 1 mg/ml collagenase P (Worthington Biochemical, USA) and 0.25% trypsin, and rotated for 10 min. Cells from this initial digestion were discarded. Fresh collagenase/trypsin solution was added and tissue fragments were rotated for 2 h at 37 °C. The solution was then centrifuged at 1000 g for 5 min. The cell pellet, consisting primarily of hPDLc, was collected and washed three times with DMEM. Cells were seeded into 6-well culture plates with completed DMEM containing 10% FBS, L-glutamine (600 µg/ml), penicillin (100 U/ml) and streptomycin (125 µg/ml); the cells were incubated in a humidified atmosphere, 95% air, 5% CO_2 at 37°C.

The medium was changed twice/week. When the cultures were preconfluent (10-14 days in culture), the cells were passed with trypsin-EDTA and placed onto 24-well culture plates; hPDL cells of the second to fifth passages were used for the experiments described below.[14]

Vitamin D treatment and MTT cell viability assay - To evaluate the concentration effect of Vitamin D (VD) on hGF and hPDL cells viability, each cell line was seeded in 0.1 ml at a concentration of 3.0×10^4 cells per well in a 96-well micro-titration plate (Corning Costar, Milan, Italy) without antibiotics in the cell medium. The medium was replaced with fresh 10% FBS-DMEM containing VD at a concentration of 10^{-10} mol/l, 10^{-9} mol/l and 10^{-8} mol/l and incubated for 12 h at 37 °C. Control cultures received fresh 10% FBSDMEM without VD. The optical density of the wells was determined using a microplate reader (Eppendorf BioPhotometer) and the viability was calculated by measuring the increase in absorbance at 570 nm and was expressed as a percentage of the control value. The MTT assay data were confirmed by counting VD-treated and VD-untreated cells in a Bürker chamber. The experiments were repeated at least three times, and the data were expressed as the mean ±SD.

Cell viability was then evaluated in hGF and hPDLc exposed or not to vitamin D at a concentration of 10^{-8} mol/l as described above and infected 100 µl of *P. gingivalis* (MOI 50) and 100 µl of *S. pyogenes* (MOI, 50). Control cultures received medium without bacteria.

To prevent overgrowth, unbound bacteria were removed by washing the plates with medium at 2 h post infection. At 8, 12 and 24 h post treatment, the supernatant (100 µl) was replaced with RPMI (without phenol red) and 50 µg of 3-[4.5-dimethyl-2.5 thiazolyl]-2.5 diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) was added to each well and incubated for 2 h at 37 °C, as described by Nagy et al.[24] The medium was removed and isopropanol was added. The optical density was measured as described above. The experiments were reapeted at least three times and the data expressed as the mean ±SD.

For the following experimental models, hGF and hPDLc exposed to 10^{-8} mol/l of VD and incubated for 12 h at 37 °C as described above will be reported as VD-treated cells.

Inhibition assay of Vitamin D on P. gingivalis and S. pyogenes growth in hGF and hPDL cells - hGF and hPDL cells were seeded in 96-well plates (Corning Costar, Milan, Italy) at a density of 1×10^6 cells/ml and were pretreated with VD at 10^{-8} mol/l for 12 h as described above. *P. gingivalis* and *S. pyogenes* cells (MOI, 50), washed and resuspended in PBS, were added and incubated in 5% CO^2 at 37 °C for 8, 12 and 24h.

After incubation, the cells were washed and lysed in cold distilled water. Bacteria were serially diluted and spread on plates for viable counts. The CFU of *P. gingivalis* and *S. pyogenes* were counted after suitable dilutions of the lysates were plated on TS agar and brain heart agar, respectively and incubated for 24-48 h at 37°C. All serial dilutions were in cell-culture medium. Bacteria were tested in triplicate and the assay repeated three times. Results were expressed as a mean of the assay ±SD.

hGF and hPDLc cytokine production in response to VD - To determine whether *P. gingivalis* and *S. pyogenes* can trigger IL-8 and IL-10 in host cells we infected hGF and hPDL cells with *P. gingivalis* and *S. pyogenes* for 8, 12 and 24h and determined the cytokine levels in the culture supernatants. The cells were collected for total RNA extraction and the supernatants for the enzyme-linked immunosorbent assay (ELISA).

In details, adherent hGF and hPDL cells grown in 24-well plates were treated with VD as above described and infected or not with *P. gingivalis* and *S. pyogenes* (MOI 50) for 8, 12 and 24h. IL-8 and IL-10 proteins were assayed from the supernatants of cultures by ELISA using titerzyme ELISA Kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis - The significance of the differences in the results of each test compared to the relative control values was determined with the Student t-test. Values of $P<0.05$ were considered statistically significant. The data are presented as means ±SD of three independent experiments.

RESULTS

MTT cell viability assay - For the dose-response experiments, hGF and hPDL cells were cultured for 12 h with 10^{-10} mol/l, 10^{-9} mol/l and 10^{-8} mol/l of vitamin D. Treatment with vitamin D significantly increased the viability rate of hGF

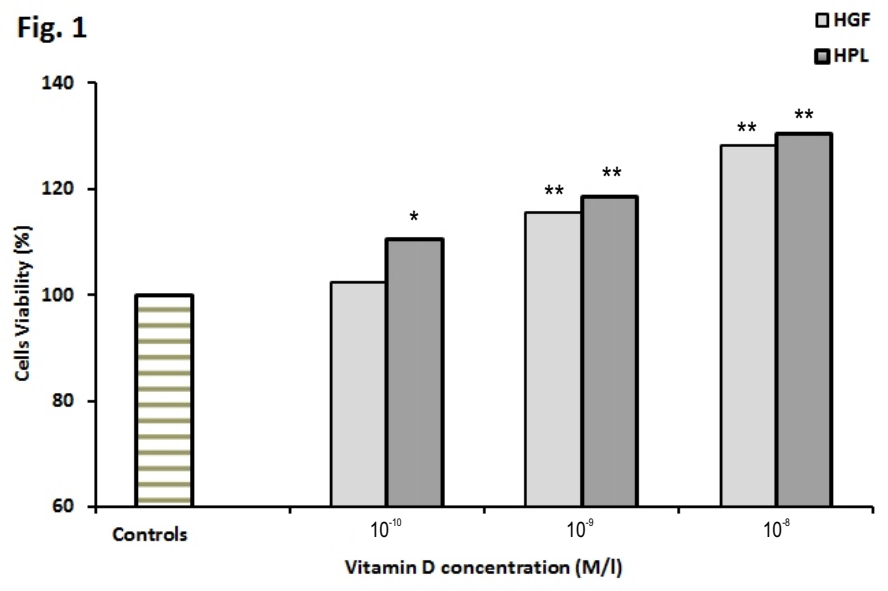
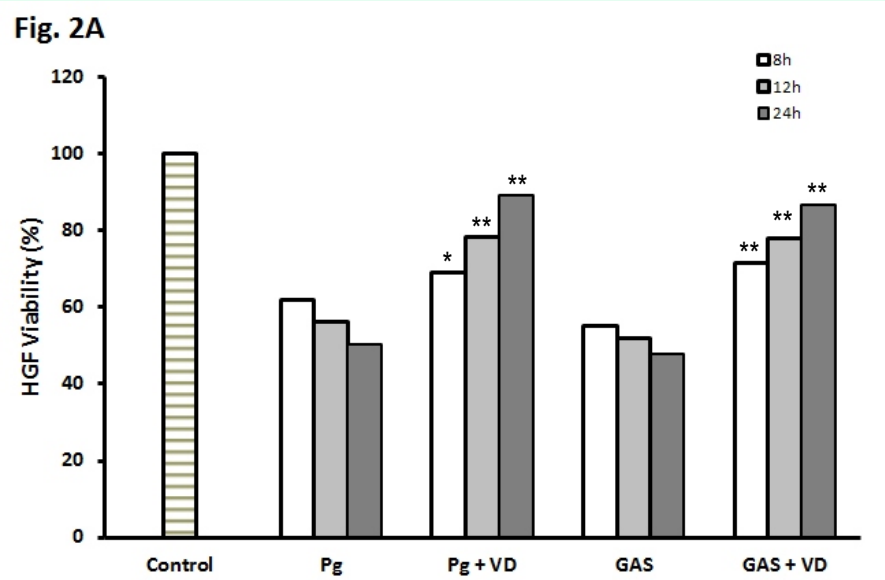


Fig.1 - Dose-dependent effect of Vitamin D 10^{-10} mol/l, 10^{-9} mol/l and 10^{-8} mol/l on cell viability rates in percent. Human gingival fibroblasts (hGF) and human periodontal ligament (hPDLc) cells were treated with Vitamin D (VD) 10^{-10} mol/l, 10^{-9} mol/l and 10^{-8} mol/l for 12 h. Data are means ± SD of three independent experiments. The asterisk indicates a statistically significant difference between the VD-treated cells and VD-untreated cells; *P < 0.05 and ** P < 0.01.

The highest viability rate was observed with a vitamin D concentration of 10^{-8} mol/l and exceed the values of untreated hGF and hPDL cells by 28.1% ± 3.9% and 30.3% ± 4.7% respectively.

In Fig. 2 we show the relationship between viability rates and time exposure to *P. gingivalis or S. pyogenes* on 10^{-8} mol/l VD treated cells. In comparison to control (no exposure to VD, no contact with bacteria) considered 100%, exposure to *P. gingivalis and S. pyogenes* leads to a reduction of the hGF cells viability rate (Fig. 2A). While the treatment with VD increased the viability of hcells after Pg and GAS infection from 50.2% ± 3.9% to 89.1% ± 4.8% and from 47.9% ± 3.5% to 86.6% ± 4.5%, respectively.

In hPDLc, treatment with vitamin D significantly increased the viability in comparison to untreated cells after *P. gingivalis and S. pyogenes* exposure times of 24 h (Fig. 2B). In particular, the treatment with VD increased hPDLc viability after Pg and GAS infection from 60.8% ± 4.2% to 94.8% ± 4.6% and from 59.5% ± 3.2% to 85.9% ± 3.8% respectively.



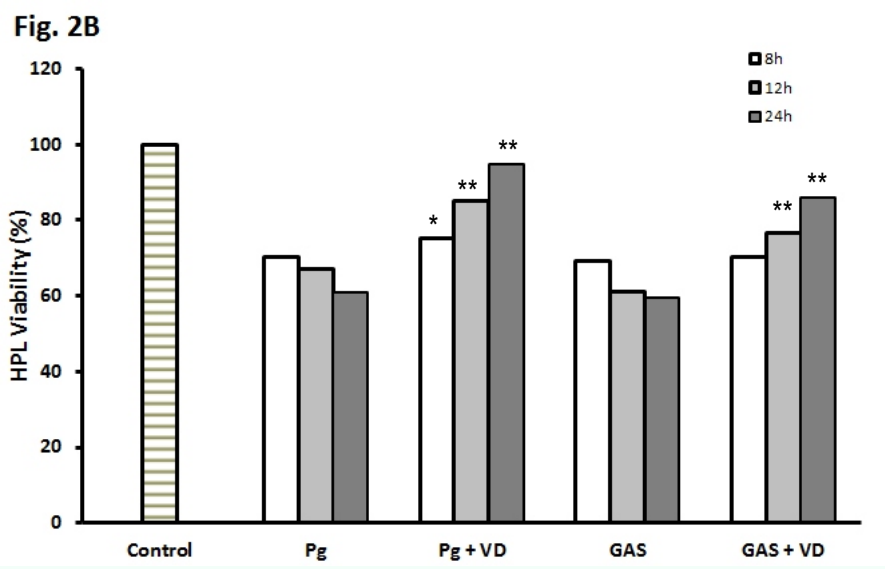
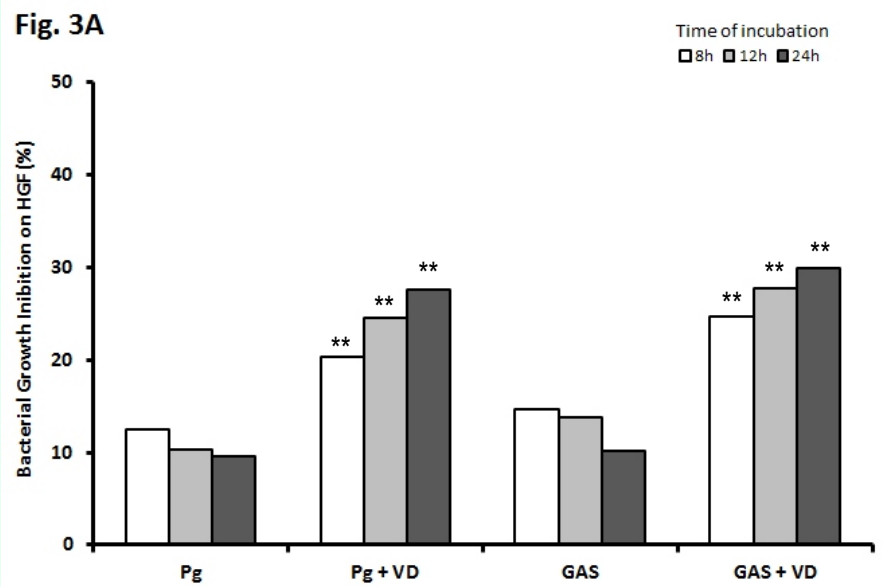


Fig.2 - Kinetics of viability rate of hGF (A) and hPDLc (B) treated with VD and exposed to *P. gingivalis* and *S. pyogenes*. hGF and hPDLc were treated with VD for 12 h and infected with *P. gingivalis* (Pg) and *S. pyogenes* [group A streptococci (GAS)] at MOI = 50 for 8, 12 and 24 h. Data are means ± SD of three independent experiments. The asterisk indicates a statistically significant difference between the infected VD-treated and untreated cells; *P < 0.05 and ** P < 0.01.

Inhibition assay of Vitamin D on *P. gingivalis and S. pyogenes* growth in hGF and hPDL cells - VD-Treated hGF and hPDLc cultures exhibited a significant increase in the antibacterial activity (Fig 3). Vitamin D showed a strong time-dependent activity against both bacteria. In particular, when VD-treated hGF cells were infected with *P. gingivalis and S. pyogenes* for 24 h, the growth inhibition was 27.6% ± 3.0% and 29.9% ± 2.8%, respectively, versus VD-untreated cells.(9.6% ± 2.5% and 10.2% ± 2.3% respectively) (Fig. 3A).

In VD-treated hPDLc infected with *P. gingivalis and S. pyogenes* for 24 h, the growth inhibition was 29.7% ± 2.5% and 32.8% ± 3.4%, respectively, compared to the 8.7% ± 2.7% and 9.5% ± 2.4% of the corresponding VD-untreated cells (Fig. 3B).



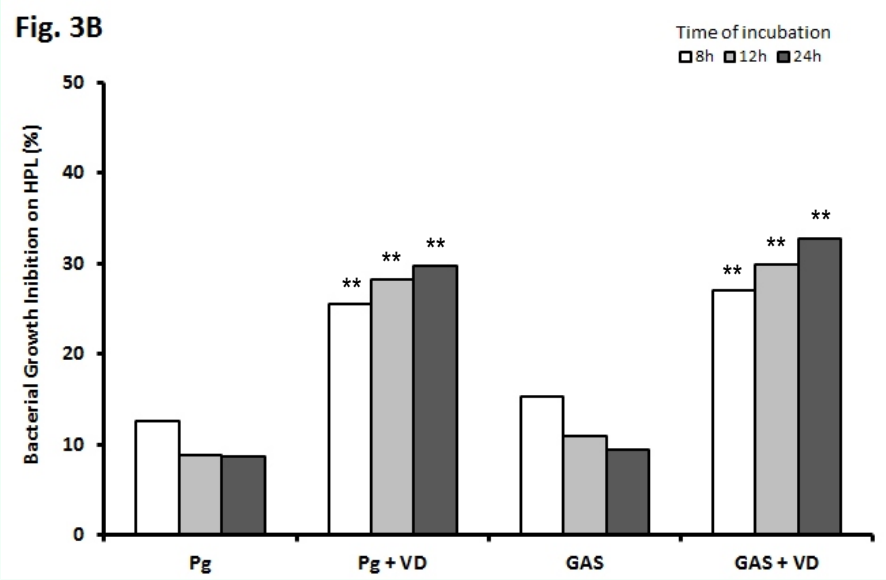
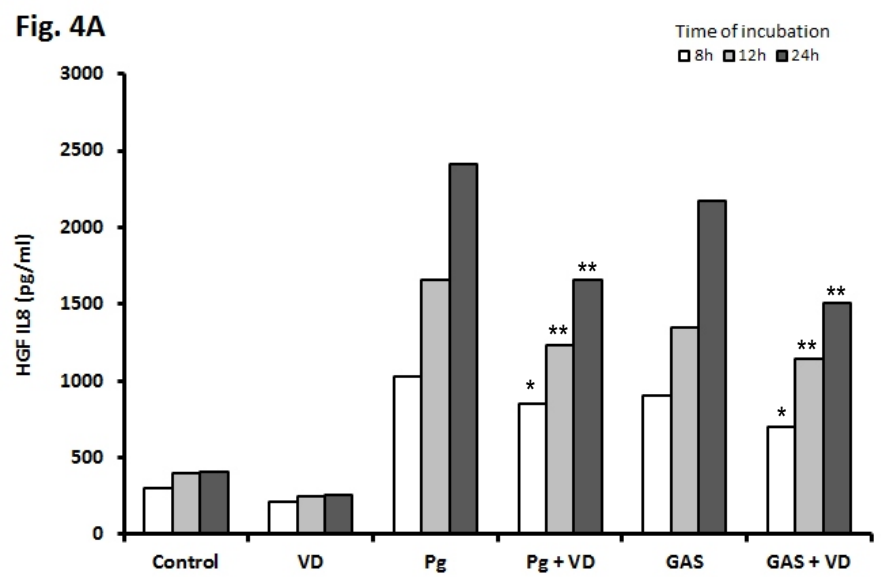


Fig.3 - Effect of VD on Pg and GAS growth inhibition in hGF (A) and hPDLc (B) (MOI = 50). hGF and hPDLc were stimulated with VD for 12h and incubated with Pg and GAS at MOI=50 for 8, 12 and 24h. Data are means ± SD of three independent experiments. The asterisks indicate a statistically significant difference between the infected VD-treated and untreated cells; *P < 0.05 and ** P < 0.01.

hGF and hPDLc cytokine production in response to VD - The levels of IL-8 and IL-10 secreted by VD-treated and VD-untreated hGF and hPDL cells were measured after the infection with *P. gingivalis and S. pyogenes*, for 8, 12 and 24 h.

hGF and hPDL cells constitutively released IL-8 (Fig. 4) at very low levels. When exposed to *P. gingivalis and to S. pyogenes* (for 8, 12 and 24 h), IL-8 levels increased in both cell types, with peak levels at 24h of bacteria exposure. However, in VD-treated hGF and hPDL cells exposed to *P. gingivalis and S. pyogenes* infection at 24h, the production of IL-8 was greatly reduced.



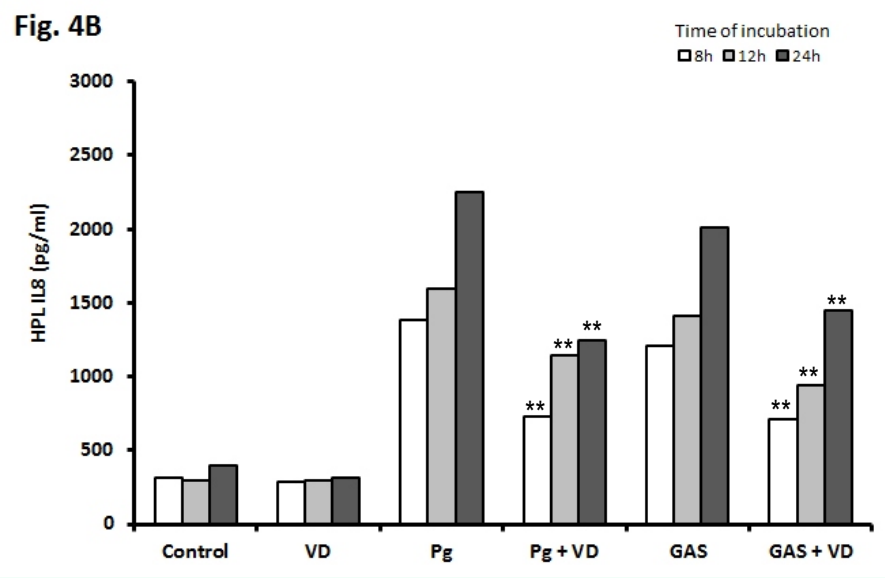
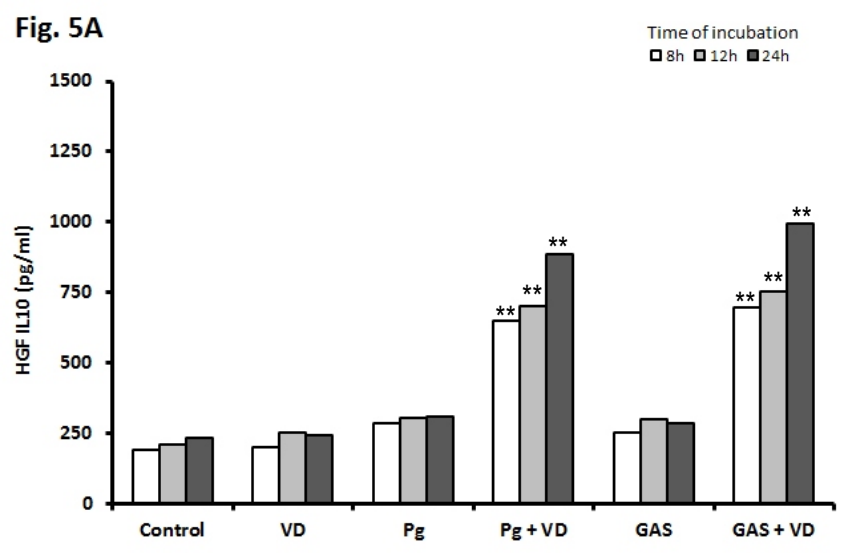


Fig.4 - IL-8 secretion in hGF (A) and hPDLc (B) cultures treated with VD for 12h and infected or not with Pg and GAS. At 8, 12 and 24h post infection, culture supernatants were collected and IL-8 was measured by ELISA. Data are means ± SD of three independent experiments. The asterisks indicate a statistically significant difference between the infected VD-treated and untreated cells; *P < 0.05 and ** P < 0.01.

Basal expression levels of IL-10 (Fig. 5) in hGF and hPDL cells were low. *P. gingivalis and S. pyogenes* infections in both cell cultures did not modify the basal level greatly.

VD-treated hGF and hPDL cells, exposed to *P. gingivalis and S. pyogenes* infections, increased IL-10 production at 24 h. In particular, in VD-treated hGF cells infected with *P. gingivalis and S. pyogenes* the IL-10 increase was 188.9% ± 15.9% and 247.9% ± 14.8% respectively compared to VD-untreated cells (Fig. 5A). Likewise, at the same experimental conditions, IL-10 increase in VD-treated hPDLc was 220.8% ± 16.3% and 221.8% ± 12.5%, respectively, compared to the VD-untreated cells (Fig. 5C).



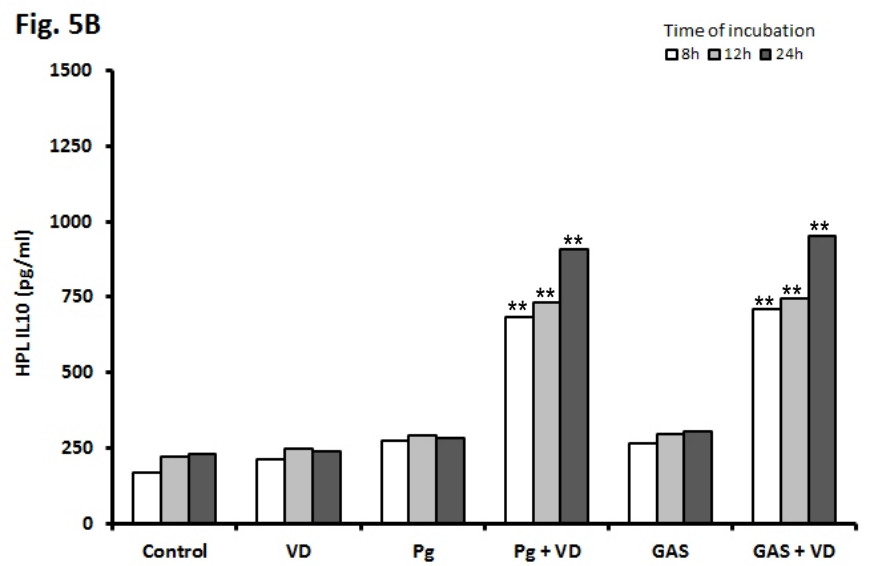


Fig.5 - IL-10 secretion in hGF (A) and hPDLc (B) cultures treated with VD for 12h and infected or not with Pg and GAS. At 8, 12 and 24h post infection, culture supernatants were collected and IL-10 was measured by ELISA. Data are means ± SD of three independent experiments. The asterisks indicate a statistically significant difference between the infected VD-treated and untreated cells; *P < 0.05 and ** P < 0.01.

DISCUSSION

Periodontal tissue homeostasis could be considered as a delicate balance between the periodontal microbiota and the host response, with occasional microbial challenges readily subdued by immune defenses. This controlled inflammatory state would represent a protective response, clinically detectable as a chronic stable gingivitis. The onset of periodontitis requires a shift in microbiological composition and a susceptible host, which exert a complex inflammatory response involving both innate and adaptative elements, that ultimately cause collateral damages to periodontal tissues. A key factor in periodontal treatment appears the ability to control or modulate reactive inflammation.

Vitamin D, which is essential for bone formation, was showed to be implicated in immunomodulatory and anti-inflammatory patterns, being its deficiency associated with cancer, infectious diseases, chronic inflammatory and autoimmune disease .[26]

In this study, we investigated the effects of vitamin D on cell viability and the capacity to modulate cytokine secretion using primary cultures of periodontal cells, both from the gingival tissues (hGF, human gingival fibroblasts), and from the periodontal ligament (hPDLc) infected with Streptococcus pyogenes and Porphyromonas gingivalis. We also evaluate the antibacterial effect of vitamin D on the two bacterial strains.

The two human cellular lines were chosen to study possible immunomodulatory effects of vitamin D under conditions resembling the in vivo situation: the first line of defense of the gingival connective tissue and the second line of defense, in the deep periodontium, where periodontal ligament cells are considered of pivotal importance in the repair of injured periodontal tissues.[27]

The cells used in the present study were all obtained from primary culture, since no established cell lines of hGF or hPDLc are available. Recently, it was reported that vitamin D might be metabolized by hGF and hPDLc and that has a positive effect on periodontal health enhancing the antibacterial defence of human gingival and periodontal cells.[28]

In the present study we showed that vitamin D increased the viability of hGF or hPDL c in culture, as demonstrated by MTT, and inhibited the *growth capacity of P. gingivalis* and *S. pyogenes on both cell cultures,* suggesting that this vitamin may affect the cellular innate immune response of hGF and hPDLc. Our data are in agreement with previous results that found that VD active form is able to inhibit the growth of *Mycobacterium tuberculosis in cultured human monocytes*.[29]

Clinical and experimental observations support the hypothesis that a deficit of vitamin D may lead to the deregulation of the human immune response and may therefore be an underlying cause of infectious diseases and immune disorders.

Here, we demonstrated that vitamin D treatment modifies the inflammatory responses of hGF and hPDLc to *P. gingivalis* and *S. pyogenes* infection. To evaluate this effect, we assayed IL-8 and IL-10 cytokine production. IL-8 is produced by a wide variety of cell types in response to bacteria and other proinflammatory mediators, such as IL-1β and TNF-α. IL-8 is a principal neutrophil chemoattractant and activator, which leads to the recruitment and infiltration of neutrophils, thereby playing a key role in inflammatory conditions. Tang et al. reported that vitamin D reduced the expression of IL-8 secreted from hPDLc, in agreement to our data.[21]

IL-10 is a cytokine expressed by most T cells including regulatory T cells, antigen-presenting cells, macrophages, epithelial cells and fibroblasts. Vitamin D promotes a shift from Th1 to Th2 cytokines, such as IL-5 and IL-10.[30] Even if anti-inflammatory properties *in vitro* vary with the cell lines used, we showed that hGF and hPDLc pre-treated with vitamin D and infected with *P. gingivalis* or *S. pyogenes* produced an increased amount of IL-10 in agreement to observation made by other authors.[31,32]

The evaluation of bacterial growth suggests that vitamin D stimulation on fibroblastic cell cultures interfere with the infective capacity of *P. gingivalis* or *S. pyogenes* by modulating cytokine responses induced by the those pathogens.

CONCLUSION

Our study supports the hypothesis that vitamin D may express multifaceted properties, including the ability to prevent and/or limit *P. Gingivalis* - or *S. Pyogenes*- induced damage, increasing cell viability and decreasing the inflammatory response, through a reduction in pro-inflammatory mediators and an increase of the protective anti-inflammatory mediators.

In conclusion, our observation that vitamin D can induce the modulation of the innate immune response suggests that this pathway can be useful for the treatment or prevention of infectious diseases in the oral cavity. The data suggest a therapeutic role for vitamin D in a variety of clinical conditions in which cytokine mediated pathology is central.

Further studies are needed to understand the molecular mechanisms by which vitamin D exerts its anti-inflammatory effects, and to evaluate the clinical implications of these findings in defining effectiveness of Vitamin D use in therapy.

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