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Original article

PDRN, a natural bioactive compound, blunts inflammation and positively reprograms healing genes in an "in vitro" model of oral mucositis

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ABSTRACT

Oral mucositis is a side effect hard to treat following high dose chemotherapy or radiotherapy. Adenosine A_{2A} receptor stimulation blocks NF-κB and boosts the Wnt/β-catenin signaling, thus blunting inflammation and triggering growth factor codifying genes. Polydeoxyribonucleotide (PDRN) is a registered drug that activates the A_{2A} receptor. Therefore, the aim of this study was to evaluate PDRN effects in an "in vitro" model of oral mucositis induced by prompting an inflammatory phenotype in human gingival fibroblasts (GF) and human oral mucosal epithelial cells (EC). GF and EC were stimulated with LPS (2 μg/ml) alone or in combination with i) PDRN (100 μg/ml); ii) PDRN plus ZM241385 (1 μM) as an A_{2AR} antagonist; iii) CGS21680 (1 μM) as an A_{2AR} agonist. LPS boosted NF-κB, TNF-α and IL-6 expression, decreased IL-10 levels and downregulated both Wnt/β-catenin, VEGF and EGF expression. PDRN reverted the LPS-induced phenotype as well as CGS21680. Co-incubation with ZM241385 abolished PDRN effects, thus confirming A_{2A} receptor involvement in PDRN mechanism of action. These results suggest that PDRN efficacy may be due to a "dual mode" of action: NF-κB inhibition and Wnt/β-catenin signaling activation. However, these interesting findings need to be confirmed by animal and clinical studies.

1. Introduction

Oral mucositis is one of the most common diseases of the oral cavity and represents an unmet therapeutic challenge for cancer patients undergoing radiation therapy and high-dose chemotherapy [1–3]. Oral mucositis has been reported in nearly 40% of patients after standard dose chemotherapy and in around 97% of patients undergoing high dose chemotherapy [4,5]. Mucosal cells have a high turnover that renders them extremely susceptible to the damage induced by ionizing radiation and chemotherapy. In fact, both therapies induce an arrest in the cell cycle machinery, in turn fostering the activation of an inflammatory cascade that exacerbates the damage and favors the triggering of apoptosis. These events lead to a rapid loss of the highly dividing mucosal cells [6]. The correlated clinical setting is characterized by erythema, ulceration and edema often associated with pain which strongly affects the quality of life of patients. This clinical scenario leads to impair nutrition with consequent weight loss, recurrent infections and increased use of analgesics until total parental nutrition and ultimately radiotherapy or chemotherapy suspension [7] in particular in patients with associated risk factors such as alcohol intake, tobacco use and poor oral hygiene [8].

The pathogenesis of oral mucositis encompasses a number of complicated events that synergize and amplify the damage. Reactive oxygen species (ROS) boost the activation of several transcription factors such as nuclear factor kappa-B (NF-κB) that represents the first step of an intracellular signaling that modulates the phenotype change of oral mucosal epithelial cells and gingival fibroblasts [6,9]. This change includes the release of several pro-inflammatory cytokine such as Tumor Necrosis Factor alpha (TNF-α and interleukin 6 (IL-6)) as well as enzymes involved in the oxidative stress (inducible NO-synthase) and

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vascular adhesion molecules in addition to a downregulation of anti-inflammatory signals, such as IL-10 [9]. Moreover, mucosal ulcers are colonized by bacteria that actively contribute to inflammation: bacterial cell wall components, such as lipopolysaccharide (LPS), stimulate pro-inflammatory cytokines secretion by macrophages [10]. This exaggerated inflammatory cascade negatively influences the intracellular signaling associated with the Wnt/β catenin pathway, an intracellular platform associated with cell proliferation and remodeling [11]. Indeed, along with its involvement in inflammation, Wnt/β catenin activates an intracellular complex signaling that conveys the triggering of genes involved in the promotion of cell growth and proliferation [12,13]. Thus, the dampening of this pathway in oral mucositis concurs to the decreased healing capacity and regeneration, for this reason Wnt/β catenin stimulation might exert curative effects.

Despite the growing interest in the management of oral mucositis, the available therapeutic interventions (sucralfate, lidocaine) for the treatment of this common disease is very poor and the obtained results are often unsatisfactory. For this reason, there is great interest to find new therapeutic strategies for the treatment of oral mucositis.

Adenosine receptors have been shown to represent an attractive target in the management of oral diseases and healing disorders [14–16]. In particular, A_{2A} receptor activation interrupts the inflammatory response and concomitantly improves tissue repair and healing process [17,18]. Our previous experiments have characterized the anti-inflammatory and tissue repair activity of a potential A_{2A} receptor agonist, Polydeoxyribonucleotide (PDRN), a biologic drug which is extracted from the gonads of trout that contains a mixture of polynucleotides [19–23]. PDRN may block NF-κB pathway and may boost the Wnt/β-catenin signaling through A_{2A} receptor activation [24,25]. In light of these evidences, the aim of this study was to evaluate PDRN effects in an in vitro model of oral mucositis induced by triggering an inflammatory phenotype in human oral mucosal epithelial cells and gingival fibroblasts.

2. Materials and methods

2.1. Cell cultures

Human primary gingival fibroblasts (GF) (atcc-pcs-pcs201–018) and human oral mucosa epithelial cells (EC) (cticc1.8.3 sk0251) were obtained from LCC Standards S.r.l Milan, Italy and Clinisciences s.r.l. Rome, Italy, respectively. Cells were put in a culture medium (DMEM, 10% fetal bovin serum, 1% antibiotic mixture) and incubated at 37 °C with 5% of CO_2. Medium was changed every 2 days and cells were replated.

2.2. Treatments

GF and EC cells were cultured in six well culture plates at a density of 2.5×10^5 cells/well and were challenged with LPS (2 μg/ml; Escherichia coli serotype 055:B5; Sigma-Aldrich, Milan, Italy) to establish an inflammatory phenotype. After stimulation with LPS, cells were treated with PDRN (100 μg/ml) (Placentex Integro, Mastelli Srl, Italy), CGS21680 (A_{2A} agonist, 1 μM) (Tocris Bioscience Bristol, United Kingdom), PDRN (100 μg/ml) + ZM241385 (A_{2A} antagonist, 1 μM) (Tocris Bioscience, Bristol, United Kingdom). ZM241385 was added with PDRN and cells were harvested after 24 h of incubation with different treatments. All doses were chosen according to previously published papers [26,27].

2.3. MTT assay

Cell viability was evaluated by MTT assay. GF and EC cells were grown and then treated with LPS (2 μg/ml) and LPS in association with different treatments. Samples were tested in a 96-well plate at a density of 8×10^4 cells/well for 24 h to evaluate the cytotoxic effect of the tested treatments. The tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Alfa Aesar, Heysham, UK) was dissolved in sterile filtered PBS and 20 μl of the mixture were added into each well 5 h before the end of the 24 h of incubation. Medium was removed and the insoluble formazan crystals were dissolved with dimethyl sulfoxide (DMSO; 200 μl/well) following 5 h. The difference between the values obtained at 540 and 620 nm of absorbance was used to calculate the average of replicates and to evaluate cytotoxicity. Results were expressed as % of cell viability compared to untreated cells and reported as means and SD [28–30].

2.4. Real time quantitative PCR amplification (RT-qPCR)

Total RNA was extracted from GF and EC cells for RTqPCR using Trizol LS Reagent (Invitrogen, Carlsbad, CA, US). Two μg of total RNA was reverse transcribed in a final volume of 20 μl using a Superscript IV (Invitrogen, Carlsbad, CA, US). cDNA (1 μl) was added to the Bright-Green qPCR Master Mix (ABM, Richmond, Canada) (20 μl per well). The final primer concentration selected to perform the analysis was 10 μM. Samples were run in duplicate and β-actin was used as an endogenous control. Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed as n-fold increase in gene expression using the CTRL group as calibrator [31–34]. Primers used for targets and reference genes are listed in Table 1.

2.5. Measurements of cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

TNF-α, IL-6 and IL-10 levels were measured in the cell culture supernatants. The products under investigation were evaluated using an Enzyme-Linked Immunosorbent Assay (ELISA) kits (Abcam, Cambridge, UK), in agreement with the instructions reported by the manufacturer. All the samples were evaluated in duplicate and the obtained results were interpolated with the pertinent standard curves. Means of the duplicated samples were used to evaluate cytokines levels and express in pg/ml [35–38].

2.6. Drugs and chemicals

The characteristics (lot numbers, manufacturers and composition) of the materials used in this study were listed in Table 2.

2.7. Statistical analysis

All data are expressed as means ± standard deviation (SD) and the values reported are the results of at least five experiments. All assays

Table 1
Primer list.

Gene	Sequence
β-actin	Fw:5'AGAGCTACGAGCTGCCTGAC3'
	Rw:5'AGCACTGTGTTGGCGTACAG3'
Wnt-1	Fw:5'CAAGATCGTCAACCGAGGCT3'
	Rw:5'AAGGTTCATGAGGAAGCGCA3'
β-catenin	Fw:5'ATGATGGTCTGCCAAGTGGG3'
	Rw:5'TCCTGGCCATATCCACCAGA3'
NF-κB	Fw:5'CCTGGATGACTCTTGGGAAA3'
	Rw:5'TCAGCCAGCTGTTTCATGTC3'
TNF-α	Fw:5'CAGAGGGCCTGTACCTCATC3'
	Rw:5'GGAAGACCCCTCCCAGATAG3'
IL-6	Fw:5'TTCGGTCCAGTTGCCTTCTC3'
	Rw:5'CAGCTCTGGCTTGTTCCTCA3'
IL-10	Fw:5'TGGCGCGGTGGATTCATAC3'
	Rw:5'AGGGGTCTGTTTTGTTGGCA3'
EGF	Fw:5'AGAGGGAGAGGATGCCACAT3'
	Rw:5'GGTTGCATTGACCCATCTGC3'
VEGF	Fw:5'AGGCCAGCACATAGGAGAGA3'
	Rw:5'ACGCGAGTCTGTGTTTTTGC3'

Table 2
Description of the materials selected for the study.

Material	Manufacturer	Lot number	Composition
DMEM	Corning	3142008	Liquid
Fetal Bovin Serum	Carlo Erba	S16960S1810	Liquid
Antibiotic	Sigma Aldrich	0000102433	Liquid
LPS	Sigma Aldrich	069M4021V	Powder
CGS21680	Tocris Bioscience	16A244341	Powder
ZM241385	Tocris Bioscience	14A40285	Powder
PDRN	Mastelli S.R.L.	602002	Liquid
MTT	Alfa Aesar	123554	Powder
DMSO	Sigma Aldrich	SHBL6823	Liquid
TRIZOL	Invitrogen	19367601	Liquid
Mastermix	ABM	0234845744001	Liquid
Superscript	Invitrogen	01016067	Liquid

were performed in duplicate to ensure reproducibility. All the variables evaluated in the study were continuous and normally distributed. The MTT assay results were expressed as % of cell viability compared to untreated cells and analyzed using one-way ANOVA with Tukey post-test for comparison between the different groups. The RT-qPCR results were expressed as n-fold increase/decrease in gene expression using the CTRL group as calibrator and analyzed using one-way ANOVA with Tukey post-test for comparison between the different groups. The ELISA results were expressed as pg/ml and analyzed using one-way ANOVA with Tukey post-test for comparison between the different groups. For all the analyzed parameters a p value < 0.05 was considered statistically significant. Graphs were prepared using GraphPad Prism (version 8.0 for macOS, San Diego, CA, US).

3. Results

3.1. PDRN does not impair cell viability

One hundred percent of viability was recorded in control cells following 24 h. PDRN did not change human gingival fibroblasts and human oral mucosal epithelial cells viability. This experimental evidence points out that the compound does not exert a cytotoxic effect (Fig. 1). Moreover, LPS challenge did not change this parameter (Fig. 1). This allows to confirm that PDRN effects are solely due to a direct modulation of the inflammatory phenotype induced by LPS stimulus.

3.2. PDRN modulates Wnt/β-catenin signaling

The Wnt/β-Catenin signaling has been clearly shown to be down-regulated during inflammation. Our results confirmed this hypothesis. In fact, LPS challenge markedly impaired Wnt/β-Catenin signaling in human gingival fibroblasts and oral mucosal epithelial cells ($p < 0.0001$ vs CTRL; Fig. 2). PDRN treatment significantly increased both Wnt-1 and β-catenin mRNA expression compared to untreated GF and EC ($p < 0.0001$ vs LPS; Fig. 2). CGS21680, a specific A_{2A} receptor agonist, also augmented Wnt-1 and β-catenin mRNA expression in GF and EC, thus confirming that A_{2A} receptor stimulation may involve Wnt/β-catenin pathway ($p < 0.0001$ vs LPS; Fig. 2). The co-incubation with the A_{2A} antagonist ZM241385 abrogated PDRN effects on Wnt-1 and β-catenin mRNA expression, thus pointing out the PDRN mode of action (Fig. 2).

3.3. PDRN reduced the inflammatory reaction

mRNA expression of the transcription factor NF-κB, of the pro-inflammatory cytokines TNF-α and IL- 6 and of the anti-inflammatory

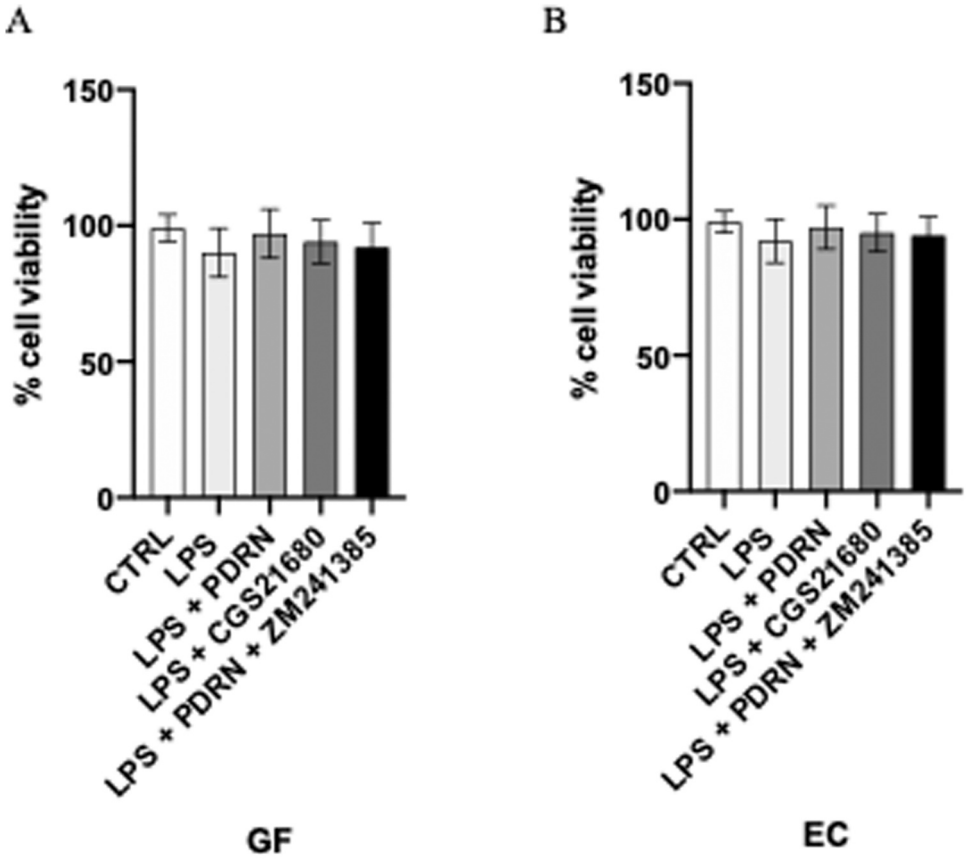

Fig. 1. The graphs show the cytotoxicity assay at 24 h in GF cells (A) and EC cells (B). Values are expressed as the means and SD.

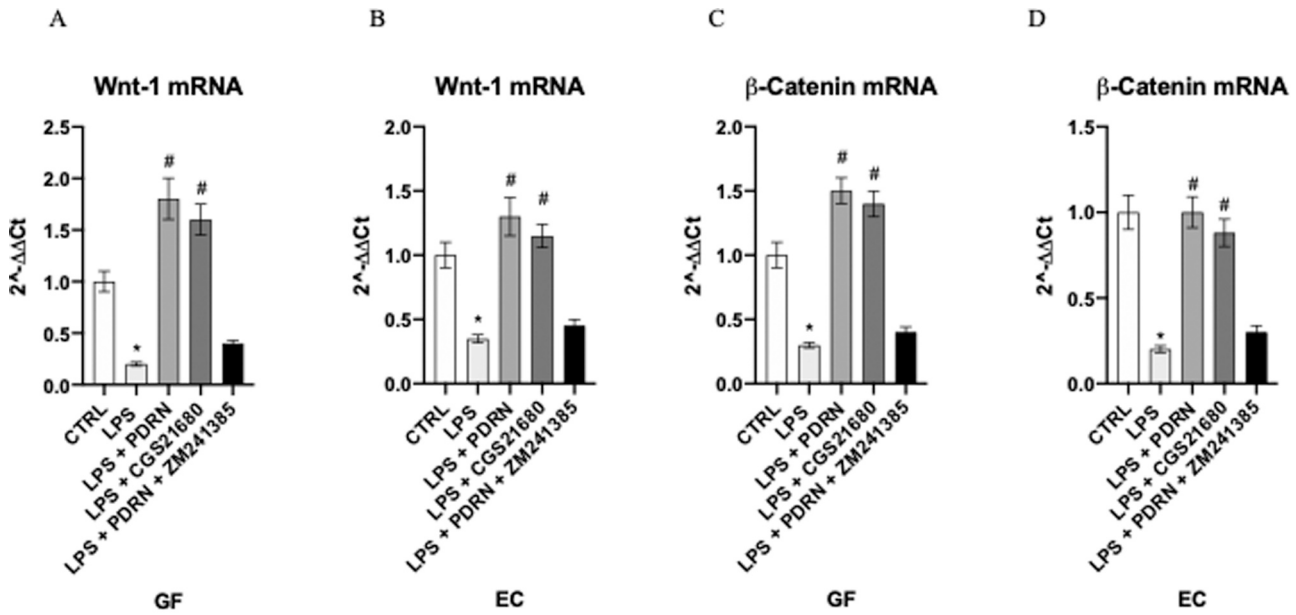

Fig. 2. The graphs represent qPCR results of Wnt-1 (A), β-Catenin (C), mRNA expression from GF cells and Wnt-1 (B), β-Catenin (D) mRNA expression from EC cells. Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS.

cytokine IL-10 was investigated to study the inflammatory panel. LPS challenge induced a significant upregulation of NF-κB, TNF-α and IL- 6 mRNA expression and a marked decrease of IL-10 expression compared to control group in both human gingival fibroblasts and oral mucosal epithelial cells ($p < 0.0001$ vs CTRL; Fig. 3 and Fig. 4). PDRN treatment blunted NF-κB, TNF-α and IL-6 and increased IL-10 mRNA expression compared to untreated GF and EC ($p < 0.0001$ vs LPS; Figs. 3 and 4). CGS21680, a specific A_{2A} receptor agonist, caused overlapping effects, thus confirming the A_{2A} receptor involvement in the modulation of the inflammatory cascade ($p < 0.0001$ vs LPS; Figs. 3 and 4). The co-incubation with the A_{2AR} antagonist ZM241385 abolished PDRN effects, thus confirming the role of the A_{2A} receptor in PDRN mode of action (Figs. 3 and 4).

Mature proteins levels were measured in the supernatants of GF and EC stimulated with LPS to confirm PDRN anti-inflammatory effect. TNF-α and IL-6 levels were markedly increased ($p < 0.0001$ vs CTRL; Fig. 5). By contrast PDRN treatment blunted TNF-α and IL-6 increase in GF and EC cells stimulated with LPS ($p < 0.0001$ vs LPS; Fig. 5).

Furthermore, IL-10 levels were markedly reduced in the supernatants of GF and EC upon LPS stimulation ($p < 0.0001$ vs CTRL; Fig. 6). PDRN treatment resulted in a significant increase of IL-10 levels ($p < 0.0001$ vs LPS; Fig. 6). CGS21680, a specific A_{2A} receptor agonist showed similar results thus confirming the anti-inflammatory effects following A_{2A} receptor activation ($p < 0.0001$ vs LPS; Fig. 6). The co-incubation with the A_{2AR} antagonist ZM241385 reverted PDRN effects thus confirming the involvement of the A_{2A} receptor activation in PDRN mechanism of action (Fig. 6).

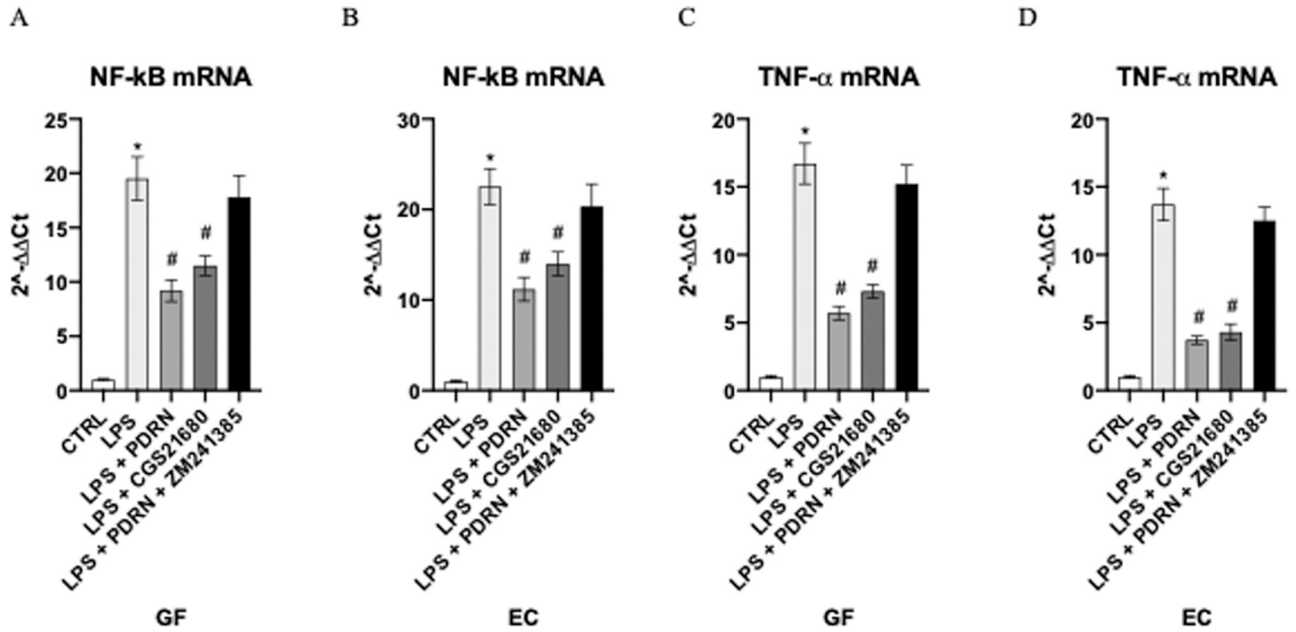

Fig. 3. The graphs represent qPCR results of NF-kB (A), TNF-α (C), mRNA expression from GF cells and NF-kB (B), TNF-α (D) mRNA expression from EC cells. Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS.

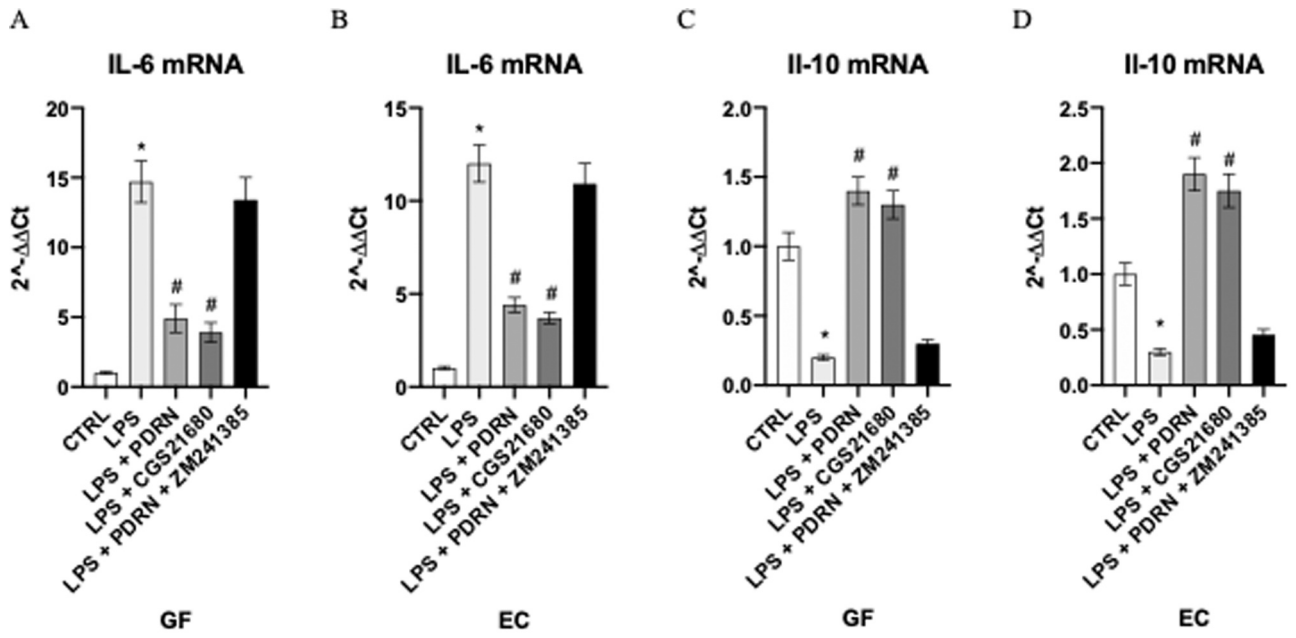

Fig. 4. The graphs represent qPCR results of IL-6 (A), IL-10 (C), mRNA expression from GF cells and IL-6 (B), IL-10 (D) mRNA expression from EC cells. Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS.

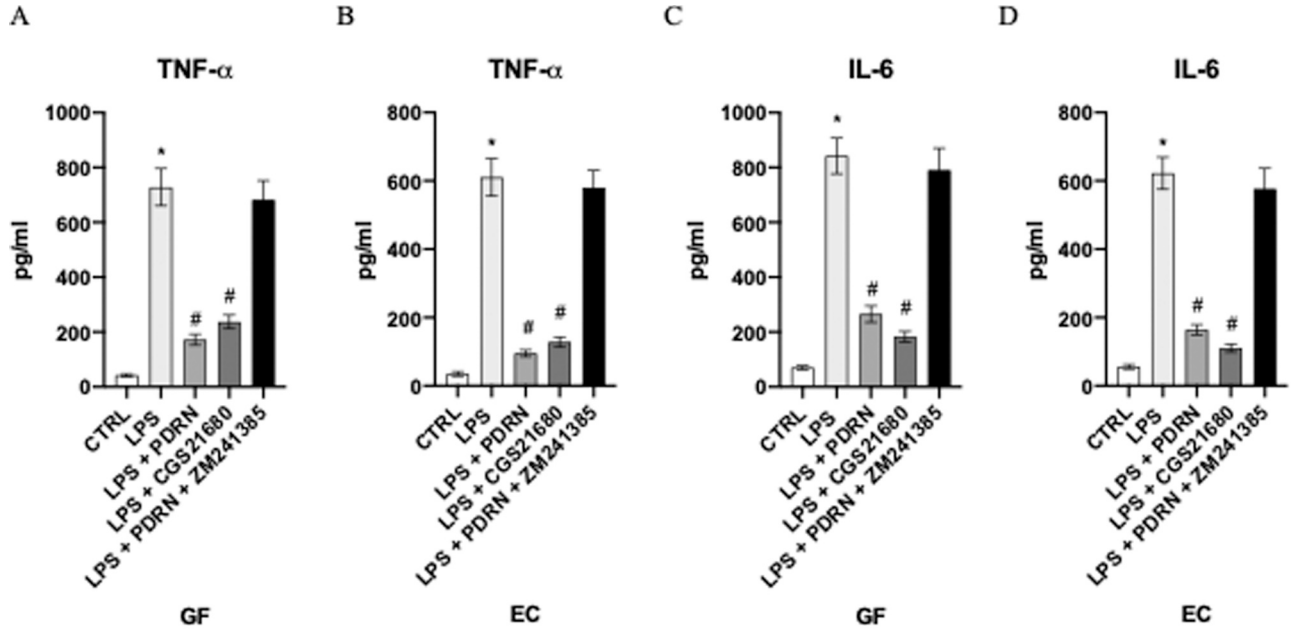

Fig. 5. The graphs represent the levels of TNF-α (A), IL-6 (C) in cell supernatants from GF cells and TNF-α (B), IL-6 (D) levels in cell supernatants from EC cells. Levels of cytokines were evaluated by immunosorbent assay (ELISA). Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS.

3.4. PDRN reprograms healing genes

LPS challenge significantly downregulated EGF and VEGF mRNA expression in both human gingival fibroblasts and oral mucosal epithelial cells compared to control group (p < 0.0001 vs CTRL; Fig. 7). PDRN treatment increased the expression of the two healing genes when compared to untreated GF and EC (p < 0.0001 vs LPS; Fig. 7). CGS21680, a specific A_{2A} receptor agonist, caused overlapping effects, thus confirming the role of A_{2A} receptor activation in the healing process (p < 0.0001 vs LPS; Fig. 7). The use of the A_{2AR} antagonist ZM241385 abolished PDRN effects, thus pointing out A_{2A} receptor involvement in PDRN mode of action (Fig. 7).

4. Discussion

This experimental study represents a "from bedside to bench-side" approach to highlight and investigate PDRN beneficial effects in oral mucositis [39]. The hypothesis was that PDRN might exert its positive activity in the setting of patients with oral mucositis, preferentially engaging the adenosine A_{2A} receptor which plays a central role in modulating inflammation, cell growth and angiogenesis. Our experimental study represents a proof-of-concept for this hypothesis. In addition, the present work unmasks for the first time an unknown role of the Wnt/β-catenin pathway in the pathogenesis of oral mucositis. Nowadays, it has been pointed out that Wnt signaling plays a central role

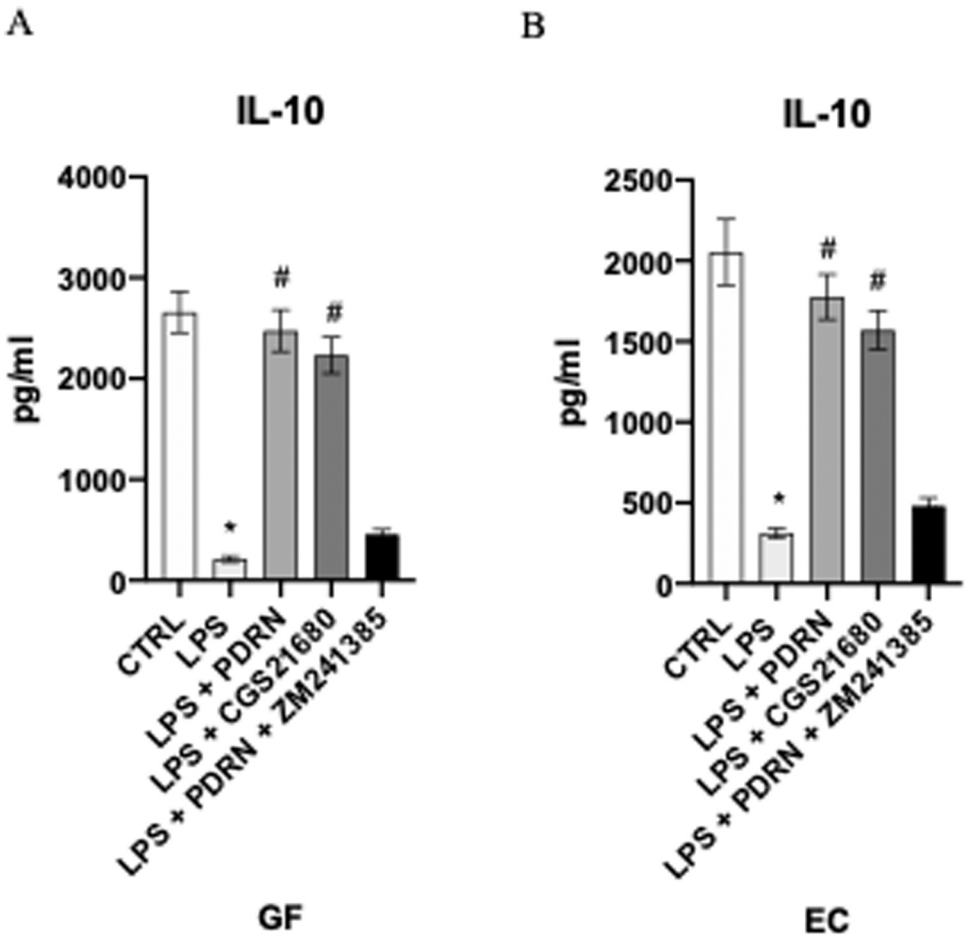

Fig. 6. The graphs represent the levels of IL-10 in cell supernatants from GF cells (A) and in cell supernatants from EC cells (B). Levels of cytokines were evaluated by immunosorbent assay (ELISA). Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS.

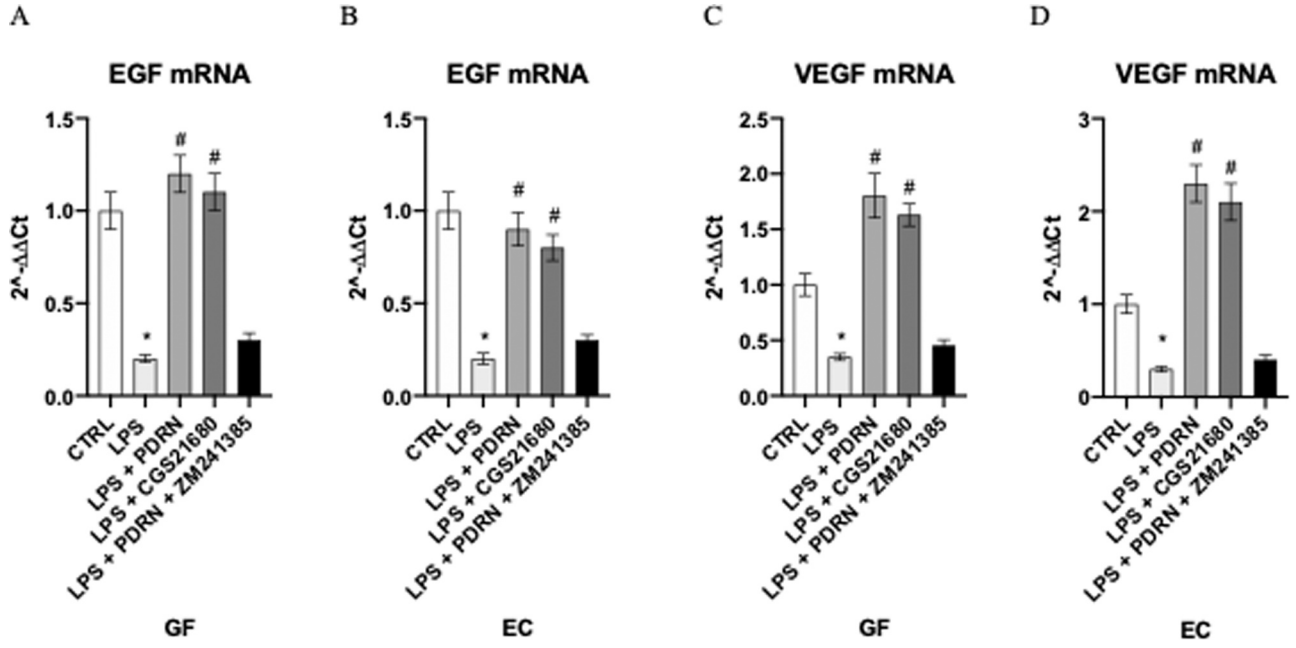

Fig. 7. The graphs represent qPCR results of EGF (A), VEGF (C), mRNA expression from GF cells and EGF (B), VEGF (D) mRNA expression from EC cells. Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS.

in several pathological conditions [40–42], moreover it is involved in a plethora of events, particularly during embryogenesis and development processes [43]. Wnt/β-catenin pathway can be triggered by A_{2A} receptor activation, in fact A_{2AR} is a G-protein coupled receptor that rises cAMP (cyclic adenosine monophosphate) levels, which is known to upregulate Wnt signaling [44]. Wnt upregulation causes β-catenin accumulation in the cytoplasm and its translocation into the nucleus, thus promoting gene transcription. In particular, it has been demonstrated that Wnt/β-catenin pathway activation modulates either immune-inflammatory mediators (IL-10) and healing genes (EGF, VEGF) [43,45]. In this experimental model, LPS challenge reduced Wnt1 and consequently β-catenin mRNA expression. Moreover, LPS caused a significant decrease of IL-10 mRNA expression, likely as a consequence of Wnt/β-catenin pathway down-regulation. Therefore "reawakening" the Wnt/β-catenin signaling may be an innovative strategy for the treatment of oral mucositis. In agreement with this experimental thesis, PDRN treatment enhanced both Wnt1 and β-catenin mRNA expression, thus restoring and boosting canonical Wnt/β-catenin pathway. In contrast, this effect was abolished by co-incubation with the A_{2A} receptor antagonist ZM241385, thus demonstrating that Wnt/β-catenin activation occurred through the adenosine A_{2A} receptor stimulation. Moreover, human gingival fibroblasts and human oral mucosal epithelial cells stimulated by LPS and treated with a specific A_{2A} receptor agonist, CGS21680, showed a significant increase of both Wnt-1 and β-catenin mRNA expression, confirming the involvement of the A_{2A} receptor in the Wnt/β-catenin pathway.

Oral mucositis is also characterized by a reduced healing that aggravates the disease clinical setting. This is due to a downregulation of genes involved in healing and re-epithelialization [46]. Previous papers showed that PDRN has regenerative and stimulating properties that are of paramount importance in the clinical setting of oral mucositis [15,16,22,23]. In agreement with these evidences, LPS challenge caused a marked downregulation of EGF and VEGF mRNA expression whereas PDRN treatment succeeded in positively reprogramming these healing genes throughout the A_{2A} receptor activation and consequently upregulation of Wnt/β-catenin pathway that lead to an increase of the transcription of genes involved in healing and re-epithelialization such as VEGF and EGF. Overlapping results were observed in GF and EC treated with CGS21680 while ZM241385 co-incubation blunted PDRN positive effects confirming again that Wnt/β-catenin activation occurred through adenosine A_{2A} receptor stimulation.

LPS stimulation also activated the transcriptional factor NF-κB and consequently promoted the expression of the proinflammatory cytokines TNF-α and IL-6 in human gingival fibroblasts and oral mucosal epithelial cells. Previous papers have been elucidated that the adenosine A_{2A} receptor activation may cause an important anti-inflammatory effect through NF-κB pathway inhibition [18,22,25]; PDRN, in fact, blunted the inflammatory response induced by LPS thanks to its activity on A_{2AR}. The present results confirmed the marked anti-inflammatory potential of this biologic compound: PDRN reduced NF-κB expression following LPS stimulation and decreased not only TNF-α and IL-6 mRNA expression but also the mature protein levels. Similar results were observed in GF and EC treated with CGS21680 while ZM241385 co-incubation dampened PDRN positive effects, confirming PDRN activity on the adenosine A_{2A} receptor. These results suggest that PDRN therapeutic effect might be ascribed to a "dual mode" of action: a stimulation of canonical Wnt/β catenin pathway that is combined with a NF-κB inhibitory activity. Thus, the A_{2A} receptor stimulation regulates two intracellular signaling cascades that result in a positive modulation of both inflammatory reactions and healing process.

Finally, it should be pointed out that PDRN is already in the market, even if, with different therapeutic indications, it is well tolerated and showed a very good safety profile [23]. PDRN half-life is approximately of 12–17 h [47], suggesting that it might be suitable for once a day dosing, easy to apply in the routine clinical practice. However, these interesting preclinical evidences deserve to be investigated in a clinical scenario of oral mucositis.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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None.

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