Suppression of Inflammatory Cytokine Secretion by an NF-κB Inhibitor DHMEQ in Nasal Polyps Fibroblasts

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Key Words
DHMEQ • Fluticasone propionate • Glucocorticoid • Nasal polyps • NF-kappaB • Rhinosinusitis • Dehydroxymethylepoxyquinomicin • In vitro culture • Polyps fibroblasts • Topical steroid

Abstract
Background: NF-κB is an essential transcription factor strongly associated to inflammatory response in chronic rhinosinusitis with nasal polyps (CRSwNP). DHMEQ is a NF-κB inhibitor that has been previously described with a great potential in decreasing inflammation in diseases other than CRSwNP. The aim of study is to evaluate the ability of DHMEQ to reduce the inflammatory recruiters on CRSwNP and to compare its anti-inflammatory profile as a single-agent or in association with fluticasone propionate (FP). Methods: Nasal polyp fibroblasts were cultured in TNF-α enriched media. Cells were submitted to three different concentrations (1, 10 and 100nM) of either FP, DHMEQ or both. Inflammatory response was accessed by VCAM-1, ICAM-1 and RANTES expression (by RTQ-PCR) and protein levels by ELISA. Nuclear translocation of NF-κB was also evaluated. Results: Both FP and DHMEQ inhibited inflammatory recruiters’ production and NF-κB nuclear translocation. Interestingly, the anti-inflammatory effect from the association steroids plus DHMEQ was more intense than of each drug in separate. Conclusion: DHMEQ seems efficient in modulating the inflammatory process in CRSwNP. The synergic anti-inflammatory effect of DHMEQ and steroids may be a promising strategy to be explored, particularly in the setting of steroid-resistant NP.
Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is an upper airway disease characterized by a remarkable recruitment of inflammatory cell line nasal mucosa [1-3], particularly eosinophils, in a mixed Th1/Th2 profile of inflammation, with a Th2 polarization [4-5]. In this process, fibroblasts, epithelial and endothelial cells are important producers of pro-inflammatory molecules (such as the adhesion molecules VCAM-1 and ICAM-1) and chemokines RANTES, eotaxin and eotaxin-2 [6-8]. Migrated inflammatory cells (such as lymphocytes and eosinophils) produce other several cytokines and chemokines, which will increase cell recruitment and survival [6, 9, 10], and thus reverberate inflammation [7].

Because of its broad effect on pro-inflammatory molecules [7], topical glucocorticoid (GC) is the medication of choice to initially treat CRSwNP [11, 12]. The GC-GR (glucocorticoid receptor) dimer acts as a negative transcription factor (TF) through the inhibition of other TFs, particularly AP-1 and NF-κB [7, 13, 14].

Recently, our group described higher expression of NF-κB in CRSwNP compared to normal nasal mucosa [15]. Moreover, higher NF-κB expression was correlated to impaired medical treatment outcome with topical glucocorticoids [16, 17]. As a result, we suggested that NF-κB expression could be an important inducer of inflammation and GC resistance for CRSwNP.

The concept of a potent anti-inflammatory drug with reduced side effects is quite an interesting concept to be chanced in CRSwNP. In this regard, dehydroxymethylepoxyquinomicin (DHMEQ) is a NF-κB inhibitor derived from the antibiotic epoxyquinomicin C [18, 19]. This drug has shown to directly inhibit the nuclear translocation and transcription of NF-κB without apparent toxic side effects [19, 20]. Since DHMEQ has previously shown to display anti-inflammatory and anti-fibrotic effects [19], and NF-κB expression was increased in CRSwNP, we considered rational to evaluate the effects of DHMEQ on CRSwNP, either alone or in association to GC.

The objective of the present study is to assess and compare the effect of DHMEQ tofluticasone propionate (FP) in reducing the expression of the pro-inflammatory molecules VCAM-1, ICAM-1 and RANTES in NP (nasal polyps) fibroblasts, either isolated or in association to this steroid.

Materials and Methods

Patients with bilateral inflammatory CRSwNP (confirmed by endoscopy and CT scans) that failed to a three-month medical treatment (including topical and systemic glucocorticoids and, if needed, short-course of antibiotics) were eligible for this study. Patients with ciliary dyskinesia, cystic fibrosis, AERD (aspirin exacerbated respiratory disease), smoking and severe asthma were excluded.

After giving informed consent, 6 consecutive patients performed a one-month-withdrawal of any medication, and NP specimens were collected during endoscopic nasal surgery. The present study was approved by Ethics local IRB (process number 4374/2007).

Cell culture

A polyp biopsy was aseptically collected, minced into 0.5mm fragments and disaggregated with collagenase type-IV for 2 hours. The fibroblasts were centrifuged, displayed in flasks supplemented with HAM-F10 (+1% penicillin, 1% streptomycin, and 20% fetal calf serum) and cultured at 37°C in a 5%CO₂ atmosphere.

After reaching 90% of confluence (one million cells/flask), cells were trypsinized and fibroblast lineage was confirmed by light microscope and flow cytometry [21]. Only cultures containing at least 95% of fibroblasts were studied.

Initially, drug toxicity was assessed, with concentrations from 1 to up 40000 nM of DHMEQ, FP or both for up to 72 hours. Cell viability was assessed through Trypan-Blue and apoptosis assays, and clonogenic assay was also performed.
For apoptosis assessment, a total of 3x10^5 cells were seeded in 25 cm^2 tissue culture flasks containing 5 mL of culture medium. After 24 h, the medium was replaced, propolis and DMSO were added, and then the cells were cultured for additional 48 h. Apoptotic cells were recognized by nuclear condensation and fragmentation, according to Lee and Shacter [22]. Treated cells were centrifuged and incubated for 5 min at 37 °C with bisbenzimide (Hoechst 33342), propidium iodide and fluorescein diacetate (Sigma Chemical Co., St. Louis, USA). Then, samples were mounted on slides, cover slipped and analyzed by fluorescence microscopy with a triple filter. Cells were scored and categorized according to differential staining: (1) normal: blue nucleus and green cytoplasm, (2) apoptotic: fragmented blue nucleus and green cytoplasm, and (3) necrotic: spherical red nucleus. 500 nuclei were analyzed per treatment.

**Cell treatment design**

After knowing the toxicity level of each drug, fibroblasts were then treated as follows:

- negative (free of drugs) and positive control (TNF-α: 25ng/mL)
- FP treatment: positive control added to: FP1 (1nM/0.45mg/mL), FP10 (10nM/4.5mg/mL) and FP100 (100nM/45mg/mL).
- DHMEQ treatment: positive control added to: DHMEQ1 (1nM/0.25mg/mL), DHMEQ10 (10nM/2.5mg/mL) and DHMEQ100 (100nM/25mg/mL).
- DHMEQ+FP: positive control added to: DHMEQ+FP1 (1nM DHMEQ and 1nM FP), DHMEQ+FP10 (10nM DHMEQ and 10nM FP) and DHMEQ+FP100 (100nM DHMEQ and 100nM FP).

To evaluate the anti-inflammatory effect, experiments were incubated for 24 hours only. Then, cells were harvested, trypsinized and stored in Trizol® (for RNA studies) and dimethyl sulfoxide (DMSO - for nuclear extraction) at -80°C. Culture media was stored at -20°C for ELISA study.

**Elisa**

Protein concentration of RANTES, sICAM-1 and sVCAM-1 was quantified in the culture medium by ELISA according to the manufacturer instructions (Biosource, CA, USA), and normalized to total protein measured by Bradford’s method.

**PCR**

RNAs were extracted with Trizol, and cDNAs were prepared with the High Capacity cDNA archive kit. The following TaqMan® (Applied Biosystems) primers and probes were used: Hs00164932 m1(ICAM-1), Hs00174575 m1(RANTES) and Hs00365486 m1(VCAM-1), in addition to the housekeeping Hs00266705_g1(GAPDH). The final primer concentration was 900 nM and the final TaqMan probe concentration was 300 nM.

Nine μL of the sample (diluted 1:10) was added to 10 μL of Universal PCR Master Mix (Applied Biosystems) and 1 μL of the probe. The plates were centrifuged for 10 seconds at 13000 rpm. All steps were carried out with the samples immersed in ice and little exposure to light. The final PCR conditions were: preheating to 50°C for 2 minutes, denaturation at 95°C for 10 minutes and 40 cycles of amplification and quantitation (15 seconds a 95°C and 60 seconds a 60°C) in 7500Real-Time PCR System® (PE Applied Biosystems). Each sample was tested in duplicate, and negative controls and calibrators were amplified in parallel to determine the efficiency of each experiment. Relative gene expression was calculated using the 2^-ΔΔCT method.

**Nuclear extraction**

To evaluate nuclear translocation of NF-kB, cell membranes were ruptured and nuclei were isolated. Cells were centrifuged at 13200rpm for 5 minutes; the pellet was washed twice in ice-cold PBS and incubated for 15 minutes with ice-cold buffer-A (10mmol/L HEPESpH7.9;10mmol/L KCl;0.1mmol/L EDTA;0.1mmol/L EGTA;1mmol/L DTT; 1mg/L aprotinin; 1mg/L leupeptin; and 1mg/L pepstatin). After cell membrane lysis, 5 μL 0.1% NP-40 was added; solution was vigorously vortexed for 1 minute, and centrifuged at 20,800g for 5 minutes at 4°C. The supernatant (cytoplasmic fraction) was discarded and the nuclear pellets were suspended in 50 μL of ice-cold buffer-B (20 mmol/L HEPES, pH 7.4;240mmol/L NaCl;0.1mmol/L EDTA;0.1mmol/L EGTA; 1mmol/L PMSE;1mmol/L DTT;1mg/L aprotinin;1mg/L leupeptin; and 1mg/L pepstatin), at 4°C for 30 minutes with periodic vortexing, centrifuged at 20,800g for 5 minutes at 4°C, and the supernatant (nuclear fraction) was stored at -80°C. The protein quantification of NF-kB was by ELISA(Total NF-kBp65, Biosource, Camarillo CA, USA).
Statistical analysis

All reactions were analyzed and compared by the Student t-test for paired samples, with the level of significance set at P<0.05. Results are presented in means and standard deviations.

Results

Fibroblast cultures

Five NP fibroblast cultures displayed more than 98% positivity for CD90 and less than 1% positive for CD34, confirming the specificity of fibroblasts composition. One sample presented only 92% of cells positive to CD90 and was excluded from the study.

Cell viability, apoptosis and clonogenic assays were tested in concentration from 1 to 40000nM of DHMEQ during 24, 48 and 72 hours. Cell viability and apoptosis assays demonstrated that more than 90% of the fibroblasts were viable and non-apoptotic up to 10000nM of DHMEQ; at the concentrations of 20000nM and 40000nM, cell viability remained between 85 and 90%, being significantly similar to control (Fig. 1). Clonogenic assay significantly decreased at the concentration of 40000nM in both analyzed periods (Fig. 2).

NP viability significantly decreased with 1000nM of FP (data not shown). As we chose for a comparative study between drugs, the studied concentrations were set at 1, 10 and 100nM of each drug.
Table 1. Protein concentrations of sICAM-1, sVCAM-1 and RANTES, after TNF-α stimulation, either alone or associated to different drugs and concentrations. Mean values and standard deviations, in folds compared to negative controls. P values when data were compared to positive controls. *: P<0.05

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>sICAM-1</th>
<th>sVCAM-1</th>
<th>RANTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>3.17±0.77</td>
<td>2.17±0.66</td>
<td>4.78±1.57</td>
</tr>
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<td>2.57±1.14</td>
<td>1.35±0.61</td>
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<tr>
<td>TNF-α + FP</td>
<td>10nM</td>
<td>2.17±0.44</td>
<td>1.29±0.72*</td>
</tr>
<tr>
<td>TNF-α + FP</td>
<td>100nM</td>
<td>1.65±0.44*</td>
<td>1.20±0.50*</td>
</tr>
<tr>
<td>DHMEQ</td>
<td>1nM</td>
<td>2.32±0.96</td>
<td>1.36±0.40*</td>
</tr>
<tr>
<td>DHMEQ</td>
<td>10nM</td>
<td>1.78±0.86</td>
<td>1.12±0.29*</td>
</tr>
<tr>
<td>DHMEQ</td>
<td>100nM</td>
<td>1.63±0.47*</td>
<td>1.06±0.30*</td>
</tr>
<tr>
<td>TNF-α + FP +</td>
<td>1nM</td>
<td>1.29±0.42</td>
<td>1.29±0.42</td>
</tr>
<tr>
<td>DHMEQ</td>
<td>10nM</td>
<td>0.92±0.34*</td>
<td>1.09±0.32*</td>
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<tr>
<td>DHMEQ</td>
<td>100nM</td>
<td>0.84±0.32*</td>
<td>0.92±0.34*</td>
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</table>

Fig. 3. Relative protein concentration of RANTES, ICAM-1 and VCAM-1 in cultures exposed to TNF-α (positive control), FP (1, 10 and 100nM), DHMEQ (1, 10 and 100nM) and FP+DHMEQ (1, 10 and 100nM of each drug). Comparison to the negative control (considered to be 1.0).

Inflammatory recruiters – protein level (Table 1 and Figs 3a to 3c)
TNF-α at 25ng/mL significantly increased protein concentrations of sICAM-1 (P=0.0075), sVCAM-1 (P=0.0173) and RANTES (P=0.0327) when compared to negative control.

FP decreased the studied proteins in a dose-dependent pattern, which was significantly different from positive control at all FP concentrations for RANTES (FP 1nM vs. TNF-α: P=0.0327), at 10nM and 100nM for sVCAM-1 (FP 10nM vs. TNF-α: P=0.0402) and at 100nM of FP for sICAM-1 (FP 100nM vs. TNF-α: P=0.0476).

DHMEQ also decreased the inflammatory recruiters in a dose-dependent pattern, which was significantly different from positive control at all DHMEQ concentrations for sVCAM-1 (DHMEQ 1nM vs. TNF-α: P=0.0146), at 10nM and 100nM DHMEQ treatments for RANTES (DHMEQ 10nM vs. TNF-α: P=0.0371) and at 100nM of DHMEQ for sICAM-1 (DHMEQ 100nM vs. TNF-α: P=0.0472). This response was statistically similar to FP.

The association of both drugs (FP and DHMEQ) led to a synergic reduction in inflammatory recruiters, which was significantly different from positive control at all
Table 2. mRNA expressions of ICAM-1, VCAM-1 and RANTES, after TNF-α stimulation, either isolated or associated to different drugs and concentrations. Mean values and standard deviations, in folds compared to negative controls. P values when data were compared to positive controls. *: P<0.05; **: P<0.01; ***: P<0.005.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>RANTES</th>
</tr>
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<tbody>
<tr>
<td>TNF-α</td>
<td>29.09±5.60</td>
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<td>TNF-α + FP</td>
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<tr>
<td></td>
<td>10nM 11.11±13.00*</td>
<td>2.19±1.24*</td>
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<td>TNF-α + DHMEQ</td>
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<td>2.38±1.23**</td>
<td>5.67±3.25</td>
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<tr>
<td></td>
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<td>2.32±1.14**</td>
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<tr>
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<td>3.41±2.42*</td>
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<tr>
<td>TNF-α + FP + DHMEQ</td>
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<td>1.61±0.82***</td>
<td>3.99±3.30*</td>
</tr>
<tr>
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<td>10nM 6.26±6.21*</td>
<td>1.35±0.95***</td>
<td>3.43±3.45*</td>
</tr>
<tr>
<td></td>
<td>100nM 4.56±5.26*</td>
<td>1.04±0.75***</td>
<td>2.57±2.57*</td>
</tr>
</tbody>
</table>

Fig. 4. Relative gene expression of RANTES, ICAM-1 and VCAM-1 in cultures exposed to TNF-α (positive control), FP (1, 10 and 100nM), DHMEQ (1, 10 and 100nM) and FP+DHMEQ (1, 10 and 100nM of each drug). Comparison to the negative control (considered to be 1.0).

DHMEQ+FP concentrations for RANTES (DHMEQ+FP 1nM vs. TNF-α: P=0.0411), and for the 10nM and 100nM DHMEQ+FP treatments for sVCAM-1 (DHMEQ+FP 10nM vs. TNF-α: P=0.0117) and for sICAM-1 (DHMEQ+FP 10nM vs. TNF-α: P=0.0205). The association of both drugs led to a statistically different response from FP alone at the concentration of 10 and 100nM for both sICAM-1 (P=0.0229) and RANTES (P=0.0409).

Inflammatory recruiters - mRNA level (Table 2 and Figs. 4a to 4c)

TNF-α 25ng/mL significantly increased mRNA expression of ICAM-1 (P=0.0004), VCAM-1 (P=0.0052) and RANTES (P=0.0312).

FP decreased gene expression in a dose-dependent pattern, which was significantly different from positive control at all FP concentrations for VCAM-1 (FP 1nM vs. TNF-α:
Table 3. Nuclear translocation of NF-kappaB after TNF-α stimulation, either isolated or associated to different drugs and concentrations. Mean values and standard deviations, in folds compared to negative controls. P values when data were compared to positive controls. *: P<0.005; **: P<0.001.

<table>
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<th>Stimulation</th>
<th>NF-kappaB</th>
</tr>
</thead>
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<tr>
<td>TNF-α</td>
<td>1.59±0.18</td>
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<td>TNF-α + FP</td>
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<tr>
<td></td>
<td>10nM</td>
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<td></td>
<td>100nM</td>
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<tr>
<td>TNF-α + DHMEQ</td>
<td>1nM</td>
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<td>100nM</td>
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<tr>
<td>TNF-α + FP + DHMEQ</td>
<td>1nM</td>
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<td>10nM</td>
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<td>100nM</td>
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</table>

Discussion

NP fibroblasts powerfully produce pro-inflammatory factors when induced by various stimuli. We observed that, when exposed to TNF-α, NP fibroblasts amplify the mRNA expression and protein secretion of VCAM-1, ICAM-1 and RANTES. Meyer et al. [23] also identified that TNF-α is a potent pro-inflammatory stimulus for NP cultures.

Silvestri et al. [6] observed, through flow cytometry, that TNF-α for 24 hours (0.5 to 20 ng/mL) induced the expression of ICAM-1, but not VCAM-1, on NP fibroblasts. Yoshifuku et al. [24] reported that TNF-α (10 ng/mL) for 24 hours was able to induce the secretion of RANTES and VCAM-1 in nasal polyp fibroblast, evaluated by ELISA. Thus, there is agreement about the fact that, in general, TNF-α induces the expression of mRNA and the protein
secretion of inflammatory factors. The data obtained in the present experiment agree with this pro-inflammatory profile of TNF-α.

TNF-α also significantly increased the nuclear translocation of NF-κB, suggesting this may be an important mechanism of induction of pro-inflammatory mediators. Ohori et al. [25] have also observed that TNF-α induced the expression of VCAM-1 in nasal fibroblasts, and this phenomenon was related to nuclear translocation of NF-κB. These results confirm that TNF-α induces the activation of this TF, inducing the expression of other pro-inflammatory genes.

Fluticasone reduced the protein secretion and gene expression in fibroblasts superexposed to TNF-α, in a dose-dependent pattern. Our results agree with those reported by Silvestri et al. [6], who observed that Fluticasone inhibited ICAM-1 secretion. Meyer et al. [23] evaluated different glucocorticoids (betamethasone and hydrocortisone), showing that they also inhibited the expression of RANTES on NP fibroblasts.

FP significantly inhibited the nuclear translocation of NF-κB in a very powerful way and even with very small concentrations of FP (1nM). This effect of fluticasone on NF-κB in nasal polyp fibroblasts had already been suggested by Silvestri et al. [6] and by our group [17], but no prior studies on nasal cell cultures have confirmed this effect to date.

The mechanism of action of glucocorticoids has not been fully elucidated. Its main anti-inflammatory effect is believed to be inhibiting pro-inflammatory TFs. DHMEQ, in turn, is the first NF-κB inhibitor that acts specifically on its nuclear translocation, and not on its secondary compounds or on the phosphorylation of its inhibitor, IκB [26]. Thus, DHMEQ is believed to be highly specific for NF-κB inhibition, with much lower rate of side effects.

There is no literature report regarding the use of DHMEQ in NP cultures. Ohori et al. [25] evaluated the effect of a different NF-κB inhibitor, MG-132, on NP fibroblasts, and observed that it significantly reduced nuclear translocation of NF-κB, as well as gene expression of VCAM-1. The authors concluded that VCAM-1 expression is induced by NF-κB, and that the use of a NF-κB inhibitor might be important strategy for treating inflammatory nasal diseases.

We have evaluated the effect of DHMEQ on gene expression and on protein secretion of inflammatory recruiters produced by fibroblasts, as well as on nuclear translocation of NF-κB. Notably, as this was essentially a pre-clinical assay, our major objective was to determine whether DHMEQ displayed a satisfactory anti-inflammatory profile on a NP model, and not to define DHMEQ concentration for therapeutical use. We observed that DHMEQ significantly reduced the secretion of sVCAM-1, RANTES and sICAM-1 in a dose-dependent pattern. This pattern of reduction was also observed at mRNA level, and also for nuclear translocation of NF-κB in NP fibroblasts, even at very low concentrations (1μM) of DHMEQ.

The reduction in nuclear translocation of NF-κB with DHMEQ was related to the decrease in VCAM-1, RANTES and ICAM-1. Yet, nuclear NF-κB reduction was much more pronounced during DHMEQ exposition than the production of pro-inflammatory molecules. Although we have not studied the influence of other TFs (like AP-1) on chemokines and adhesion molecules, this could be a reasonable explanation for this particular finding.

It is important to stress that the DHMEQ concentrations exploited in the present study were much lower than the doses used in previous experiments in other cultures [26, 27], as the concentration found to be toxic for NP fibroblasts cultures (40μM). Yet, these reduced doses of DHMEQ were chosen because our initial goal was to compare the effect of this drug to Fluticasone. Higher concentration of fluticasone in NP culture could lead to cell toxicity and reduction of cell viability [23].

Since the inflammatory inhibition of glucocorticoid is not limited to NF-κB pathway, we have also investigated whether the two drugs combined could potentiate their anti-inflammatory effect. This information is especially important because association DHMEQ/topical glucocorticoid could not be only synergic in potency, but also could decrease the concentration of each drug necessary to achieve anti-inflammatory pattern, and finally reduce the resistance to glucocorticoid.

In general, the present study showed that the association DHMEQ/topical glucocorticoid reduced more pronoucedly the inflammatory recruiters, and lower drug concentrations of
each drug were necessary to obtain the same anti-inflammatory results of monotherapy. In some concentrations, however, improvement was not significantly different.

Reports on the association of DHMEQ and other anti-inflammatory drugs are very scarce in the literature. Ueki et al. [27] studied the effect of DHMEQ and tacrolimus to inhibit the rejection of allogeneic heart transplant in rats, and concluded that the combined inhibition of NF-κB (by DHMEQ) and NFAT (by tacrolimus) strongly suppressed organ rejection, and thus improved survival rate and quality of life. Our results also point to an interesting rationale for the use of DHMEQ as an inhibitor of inflammatory mediators in NP, either separately or in combination with glucocorticoids.

This study provides the molecular bases for a new therapeutic strategy with a specific NFκB-targeting molecule to be evaluated in CRSwNP patients. Future studies are needed to determine the ideal doses and schedules of DHMEQ in CRSwNP patients, as well as its efficacy on controlled clinical trials for this disease.

Acknowledgements


References