

## Dexamethasone-induced apoptosis of freshly isolated human nasal epithelial cells concomitant with abrogation of IL-8 production\*

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### SUMMARY

**Background:** Human nasal epithelial cells (hNECs) are the first line of immune defense and are able to produce mediators that recruit, activate and prolong survival of immune cells, among which IL-8 takes an important place. This study investigates the contribution of freshly isolated hNECs to IL-8 production in chronic rhinosinusitis with nasal polyps (CRSwithNP). Secondly, the effects of dexamethasone treatment on hNEC apoptosis and IL-8 production are investigated.

**Methodology:** hNECs were isolated from nasal polyps and healthy inferior turbinate of NP patients and from inferior turbinates of healthy donors by protease treatment and two negative selection procedures. hNECs were incubated with IL-1 $\beta$ , TNF- $\alpha$  or dexamethasone. After 24h, IL-8 levels were determined in the supernatants by ELISA. Finally, hNECs were incubated with increasing doses of dexamethasone and apoptosis was studied.

**Results:** hNECs isolated from nasal turbinates of healthy and NP patients and polyp tissue from NP patients produced similar levels of IL-8. IL-1 $\beta$  induced higher levels of IL-8 production in all types of hNECs without differences between control and NP tissue. Dexamethasone induced apoptosis of hNECs concomitant with abrogation of IL-8 production by hNECs.

**Conclusions:** IL-8 production by human nasal epithelial cells does not differ between NP and healthy tissue under baseline nor stimulatory conditions. Dexamethasone induces apoptosis of hNECs and abrogates IL-8 production.

**Key words:** IL-8, human nasal epithelial cells (hNECs), chronic rhinosinusitis with nasal polyps (CRSwithNP) and dexamethasone

### INTRODUCTION

Epithelial cells lining the nasal cavity have a pivotal role in detecting and responding to various environmental stimuli. Nasal epithelial cells are more than just a physical barrier. They exert their immunological function by producing various cytokines and growth factors that enable the recruitment and effector function of immune cells<sup>(1)</sup>. In addition, supernatants from cultured nasal or airway epithelial cells have been shown to prolong the survival of neutrophils<sup>(2)</sup> and eosinophils<sup>(3)</sup>. Many authors have studied airway epithelial cell biology using either immortalized cell lines<sup>(1,4-6)</sup> or freshly isolated nasal/airway epithelial cells. In most studies, primary epithelial cells obtained by positive selection are grown for 6-14 days in a medium rich in growth factors and mediators that stimulate growth of epithelial cells and apoptosis of other cell types<sup>(2,6-9)</sup>.

Although these techniques yield 99,5% pure epithelial cell cultures, long term incubation might introduce immunological alterations in parallel with variations in phenotypical aspects of epithelial cells<sup>(10-12)</sup>. Here we build further on a recently reported novel technique of isolation and purification of primary nasal epithelial cells (hNECs) using two negative selections<sup>(13)</sup>. Epithelial cells were freshly isolated and incubated to study the contribution of human nasal epithelial cells (hNECs) to IL-8 production in chronic rhinosinusitis with nasal polyps (CRSwithNP).

IL-8 is one of the major cytokines secreted by epithelial cells upon stimulation. It is mainly produced upon stimulation of the toll-like receptors (TLRs) on the surface of the epithelial cells by an antigen<sup>(14)</sup> or microbial products. Super-antigens *Staphylococcus aureus* enterotoxin A (SEA) and B (SEB)<sup>(15)</sup> and pro-inflammatory cytokines such as IL-1 $\beta$ <sup>(5,16)</sup>, TNF- $\alpha$ <sup>(8,16)</sup> and IL-17<sup>(17,18)</sup> can

induce or modulate IL-8 production by human epithelial cells. Endothelin-1<sup>(19)</sup> and VIP<sup>(20)</sup> were also shown to be potent inducers of IL-8 in human nasal epithelial cells. IL-8 acts through the G protein-coupled serpentine receptors CXCR1 and CXCR2 and its primary function is the recruitment of neutrophils to the site of inflammation. It is also known as neutrophil-chemotactic factor or CXCL8<sup>(21)</sup>.

CRSwithNP is characterised by a Th2 type inflammation associated with high levels of IL-8 and neutrophils in NP tissue<sup>(22)</sup>. It has been shown that IL-8 is up-regulated in CRS patients with NP being mainly produced by epithelial cells and eosinophils<sup>(23,24)</sup>. IL-8 is the major chemo-attractant factor for neutrophils, but the precise contribution of neutrophil influx in NP disease remains obscure. By releasing TGF- $\beta$ <sup>(25)</sup> and matrix metalloproteinase 9<sup>(26)</sup>, neutrophils contribute to the reorganization of the extracellular matrix and the pathology of NP disease.

In this study, we aimed to investigate the effect of dexamethasone on nasal epithelial cell survival and IL-8 production. Dexamethasone represents a corticosteroid with broad anti-inflammatory potential. The use of corticosteroids in the treatment of NP disease is wide-spread as it is the only currently available treatment option with proven efficacy in NP<sup>(27)</sup>. Dorscheid et al. described dexamethasone-induced apoptosis of airway epithelial cells, in both primary and immortalized cell lines<sup>(6)</sup>. In addition, it has been shown that dexamethasone reduces the levels of IL-8 in human airway epithelial cells by different mechanisms: up-regulating glucocorticoid-induced leucine zipper (GILZ) which inhibits the transcription of NF- $\kappa$ B<sup>(28)</sup>, by inhibiting the binding of NF- $\kappa$ B transcription factor to its responsive elements<sup>(29)</sup> or by destabilizing the IL-8 mRNA transcript<sup>(30)</sup>. In addition, bacterial products such as LPS and peptidoglycans increase the activity NF- $\kappa$ B in paranasal sinus epithelial cells which is suppressed by preincubation with dexamethasone<sup>(31)</sup>. However, there are reports of corticosteroid treatment inducing higher production of IL-8 in airway epithelial cells of asthmatic patients together with neutrophilic airway inflammation being associated with unresponsiveness to corticosteroid therapy<sup>(32)</sup>.

## METHODS

### Patients' characteristics

Nasal biopsies were taken from patients during surgery, after a written informed consent was obtained. A biopsy of the inferior

	Control patients (n = 19)	NP patients (n = 12)
Females	7/19 (37%)	6/12 (50%)
Nasal corticosteroids	7/19 (37%)	10/12 (83%)
Atopy	7/19 (37%)	6/12 (50%)
Asthma	1/19 (5%)	5/12 (42%)
AIA	0/19 (0%)	2/12 (17%)
Current smokers	4/19 (21%)	3/12 (25%)

\* Patients are characterized by the presence of atopy, asthma (or AIA – aspirin-induced asthma syndrome), smoking habits and usage of nasal corticosteroids.

turbinate and a piece of nasal polyp tissue were taken during surgery from CRS patients with nasal polyps (CRSwithNP, n = 12), while a biopsy of the inferior turbinate was obtained from healthy control patients (n = 19) without endoscopic evidence of CRSwithNP who underwent surgery for anatomical or aesthetical reasons. Patients' characteristics are listed in Table 1. The diagnosis of atopy was based on positive skin prick test results for a panel of 18 common inhalant allergens (HAL Allergy, Leiden, The Netherlands). The diagnosis of aspirin-intolerance and asthma were history based. The study was approved by the ethical committee of the Catholic University Hospital Leuven.

### Isolation and stimulation of nasal epithelial cells (NECs)

A highly purified epithelial cell population was obtained by the following procedure (Figure 1) as reported previously<sup>(13)</sup>. Tissue was washed in sterile saline and enzymatically digested in 0.1% pronase (Protease XIV, Sigma) solution in culture medium (Lonza BioWhittaker DMEM) supplemented with L-Glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2% (v/v) ultrosor G. After overnight incubation at 4°C with shaking, the protease reaction was stopped by the addition of FCS (1:10 (v/v)). Cells were washed in culture medium and pelleted by centrifugation for 5 min on 800 rpm. Washed cells were re-suspended in 10 ml culture medium and incubated in a plastic culture flask for 1 h at 37°C in a cell incubator to remove the fibroblasts. The cell suspension was mixed with 2 x 10<sup>7</sup> prewashed CD45-magnetic beads (Dynabeads<sup>®</sup> CD45, Invitrogen) and epithelial cells were purified by the negative selection following the manufacturer's instructions (Figure 1). Cell purity was verified by cytospin preparations and was found to be  $\geq$  98%. The final cell suspension was centrifuged and re-suspended in 1 ml culture medium.

### Epithelial stimulation procedure

Purified human nasal epithelial cells (hNECs) were counted in the Bürker's chamber and different numbers of hNECs (1 x 10<sup>5</sup>, 5 x 10<sup>5</sup>, 1 x 10<sup>6</sup>) were incubated in 1 ml culture medium with or without IL-1 $\beta$  (10 ng/ml) and TNF- $\alpha$  (10 ng/ml) for 24 h at 37°C. Pilot studies involving different concentrations of IL-1 $\beta$  and TNF- $\alpha$  showed that 10 ng/ml was the optimal dose for stimulation of hNECs (data not shown). Additionally, three different concentrations of hNECs (1 x 10<sup>5</sup>, 5 x 10<sup>5</sup> and 1 x 10<sup>6</sup> cells/ml) were incu-

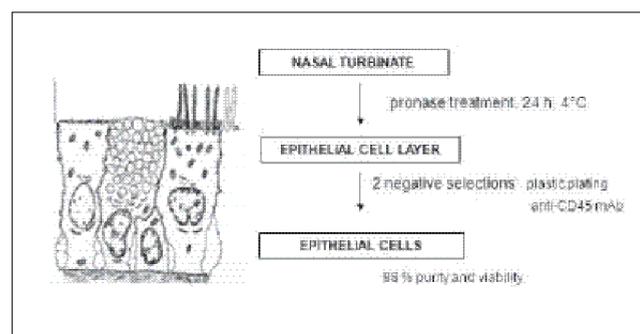


Figure 1. Purification procedure of human nasal epithelial cells (hNECs).

bated with increasing doses of dexamethasone (10, 100, 1000  $\mu$ g/ml) for 24 h at 37°C.

#### Evaluation of IL-8 production

After 24h incubation of hNECs in different conditions with/without pro-inflammatory cytokines or dexamethasone, supernatants were harvested and stored at -20°C until subsequent analysis. IL-8 was measured in the supernatants of hNECs by sandwich ELISA (capture antibody G265-5 (554716), biotinylated detection antibody G265-8 (554718), rhIL-8 as standard (554609), BD Pharmingen™, BD Bioscience).

#### Apoptosis assay

In a separate series of experiments,  $1 \times 10^5$  cells/ml of freshly isolated hNECs from inferior turbinate of four healthy donors were incubated with increasing doses of dexamethasone (10, 100, 1000  $\mu$ g/ml) or medium for 6h or 24h on 37°C. Cells were subsequently washed and the number of viable cells was determined by trypan-blue staining after 24h. To estimate the degree of apoptosis, cells were stained with annexin V-FITC and propidium-iodide (PI) following the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit I, 556547, BD Pharmingen™, BD Bioscience) after 6h of incubation. Namely, hNECs were washed twice with PBS and re-suspended in 1X Annexin V binding buffer at a concentration of  $1 \times 10^6$  cells/ml. One hundred  $\mu$ l of cell suspension was transferred to a 5 ml culture tube and 5  $\mu$ l of annexin V-FITC and 5  $\mu$ l of PI were added. Cells were incubated with annexin V-FITC and PI for 15 min at RT in the dark. Finally, 400  $\mu$ l of 1X binding buffer was added to each tube. Five hundred  $\mu$ l of cell suspension was placed on the microscopic slide by cytospin technique and visualized under the laser scanning confocal microscope LSM 510 (Carl Zeiss) with a 40x magnification lens. The FITC-labeled annexin V appeared green and the propidium iodide-labeled nuclei appeared red. The images were analyzed using LSM image examiner software (Carl Zeiss). Four different fields were counted independently and stained cells were differentiated into annexin-FITC+PI- cells (early apoptotic cells) and annexin-FITC±PI+ (late apoptotic or already dead cells).

#### Statistical analysis

Statistical analysis was performed with GraphPad software Prism 4 (<http://www.graphpad.com/prism/Prism.htm>). All outcome variables were compared using non-parametrical tests. The Mann Whitney-U test was used for between-group comparison. For stimulation experiments and dose-dependent within-group comparison Wilcoxon signed-rank test was performed. A difference was considered to be significant when  $p < 0.05$ . Data are expressed as mean with error bars expressing standard error of the mean.

## RESULTS

#### IL-8 production by freshly isolated human nasal epithelial cells (hNECs) stimulated with/without IL-1 $\beta$ and TNF- $\alpha$

Nasal epithelial cells were freshly isolated from inferior turbinate of control and NP patients as well as from the polyp tissue taken

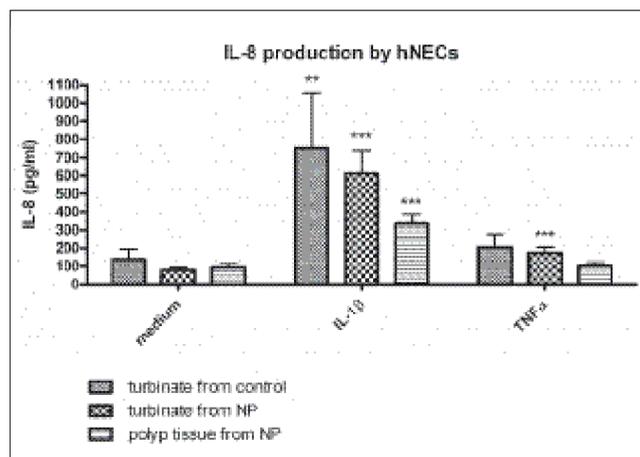


Figure 2. Effects of pro-inflammatory cytokines on IL-8 production. Production of IL-8 by hNECs upon incubation with pro-inflammatory cytokines IL-1 $\beta$  and TNF $\alpha$  is compared to the medium. Levels of IL-8 are expressed in pg/ml as measured by ELISA in the supernatants of hNECs cultures. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to medium condition of the same patient group. Control patients  $n = 13$ , NP (nasal polyp patients)  $n = 12$ .

from NP patients. Human NECs ( $1 \times 10^5$  cells/ml) were incubated in medium for 24h in order to evaluate the basal IL-8 levels in supernatants. IL-8 was detectable in all experimental conditions without significant differences in IL-8 levels between the three groups (Figure 2).  $1 \times 10^5$  cells/ml hNECs were stimulated with pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  for 24h to evaluate IL-8 production. Stimulation with IL-1 $\beta$  induced significantly higher absolute amounts of IL-8 in human NECs in all three groups (control turbinate  $136.9 \pm 57.77$  vs.  $752.2 \pm 300.2$ ,  $p < 0.01$ ; turbinate from NP  $77.83 \pm 15.37$  vs.  $610.8 \pm 127.6$ ,  $p < 0.001$ ; polyp tissue  $93.85 \pm 22.25$  vs.  $335.1 \pm 50.38$ ,  $p < 0.001$ , Figure 2). TNF- $\alpha$  had less effect on IL-8 production and significant up-regulation of IL-8 by TNF- $\alpha$  was seen only in hNECs isolated from nasal turbinate of NP patients.

In addition, we also evaluated whether allergy or usage of nasal steroids would affect IL-8 production by hNECs. Patients who are allergic or who were using nasal steroids did not show any difference in IL-8 production under baseline conditions or when stimulated with pro-inflammatory cytokines compared to non-allergic and non-steroid using patients regardless of the tissue source (data not shown).

#### IL-8 production by hNECs in response to dexamethasone

Dexamethasone is a potent anti-inflammatory drug of the steroid family. To investigate the direct effect of dexamethasone on IL-8 production by NECs, we incubated different numbers of freshly isolated hNECs with three different doses of dexamethasone (10, 100, 1000  $\mu$ g/ml). Dexamethasone at low dose (10  $\mu$ g/ml) did not significantly alter IL-8 production by hNECs isolated from NP and control patients (in control patients for  $1 \times 10^5$  cells/ml IL-8 levels in the medium are  $139.1 \pm 77.30$  vs.  $307.1 \pm 214.7$  in the condition with lowest dexamethasone concentration,  $p = 0.426$ ; for  $5 \times 10^5$

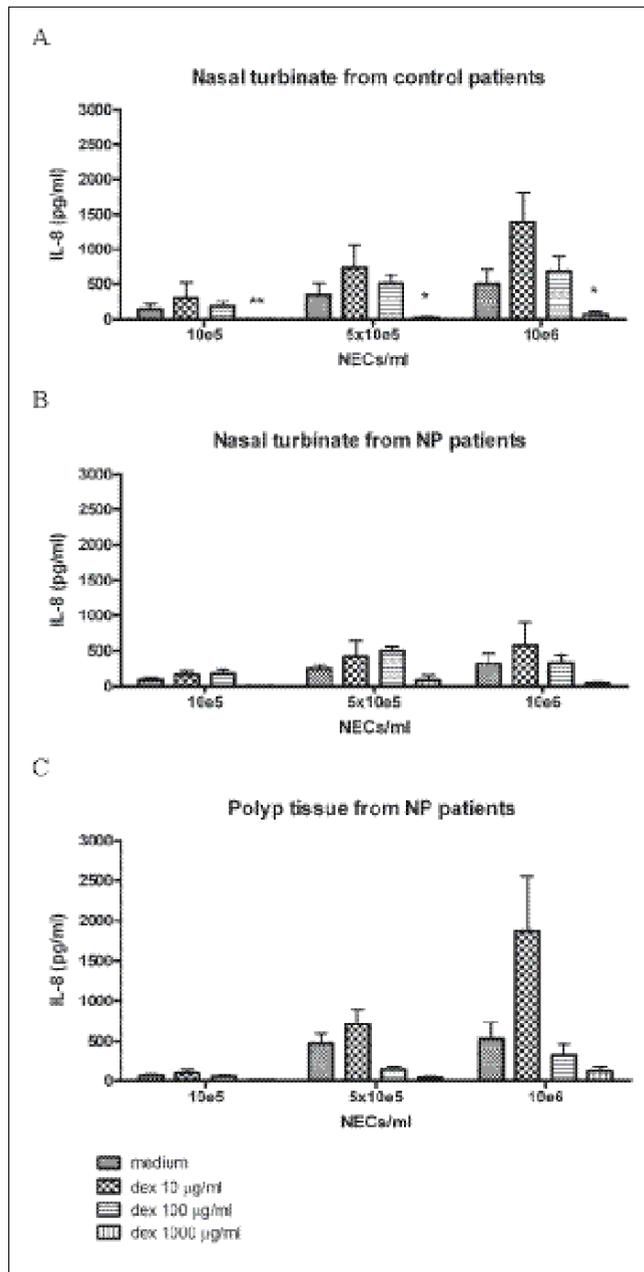


Figure 3. Effect of dexamethasone on IL-8 production. Production of IL-8 by hNECs upon stimulation with three different doses of dexamethasone (10, 100, 1000  $\mu\text{g/ml}$ ) is compared to the medium. The highest dose of dexamethasone significantly down-regulated IL-8 production in the hNECs isolated from nasal turbinate of control patients (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) compared to the medium. A) hNECs isolated from inferior turbinate of control patients ( $n = 8$ ), B) hNECs isolated from inferior turbinate of NP patients ( $n = 3$ ), C) hNECs isolated from nasal polyp tissue taken from NP patients ( $n = 5$ ).

cells/ml  $349.1 \pm 162.2$  vs.  $740.8 \pm 324.7$ ,  $p = 0.156$ ; for  $1 \times 10^6$  cells/ml  $502.2 \pm 216.9$  vs.  $1390 \pm 429.5$ ,  $p = 0.156$ ; Figure 3 A). In contrast, the highest dose of dexamethasone (1000  $\mu\text{g/ml}$ ) completely abolished IL-8 production by hNECs (in control patients for  $1 \times 10^5$  cells/ml IL-8 levels in the medium are  $139.1 \pm 77.30$  vs.  $3.633 \pm 1.305$  in the condition with highest dexamethasone concen-

tration,  $p = 0.004$ ; for  $5 \times 10^5$  cells/ml  $349.1 \pm 162.2$  vs.  $22.13 \pm 14.94$ ,  $p = 0.016$ ; for  $1 \times 10^6$  cells/ml  $502.2 \pm 216.9$  vs.  $63.29 \pm 44.87$ ,  $p = 0.031$ ; Figure 3 A). This finding was significant only for hNECs isolated from healthy nasal turbinate (Figure 3 B and C).

When increasing numbers of hNECs were incubated with the same dose of dexamethasone, we observed the same trends in IL-8 production in response to dexamethasone in all hNECs. This was observed in hNECs isolated from either polyp tissue, inferior turbinate from both NP and control patients (Figure 3 A-C).

#### Induction of apoptosis of hNECs by dexamethasone

In the previous experiment it was shown that high doses of dexamethasone decreased IL-8 production by hNECs whereas low doses did not alter IL-8 levels. We speculated that this effect is due to the induction of apoptosis of hNECs by dexamethasone. To prove that dexamethasone has a pro-apoptotic effect on hNECs, freshly isolated hNECs were incubated with different doses of dexamethasone and stained with trypan-blue to estimate the cell viability and with annexin V-FITC and PI to evaluate the degree of apoptosis. Trypan-blue staining of  $1 \times 10^5$  cells/ml freshly isolated hNECs from control turbinates showed loss of cell viability with increasing doses of dexamethasone (Figure 4 A), indicating that hNECs undergo faster cell death in the presence of dexamethasone. This finding was additionally supported by the annexin V-FITC/PI staining (Figure 4B and C). hNECs incubated with medium alone or low doses of dexamethasone (10  $\mu\text{g/ml}$ ) had more annexin V-FITC+ PI- cells indicating an early apoptotic stage, whereas numbers of annexin V-FITC+PI+ cells (late apoptotic cells) increased with the higher concentrations of dexamethasone (Figure 4 B and C).

#### DISCUSSION

We here present a novel technique for studying human nasal epithelial cells in vitro which enables investigating the effects of different pro-inflammatory stimuli on hNECs and dissecting their contribution to NP disease. This technique overcomes the problems with immortalized cell lines whose features and immunological responses may significantly differ from primary cell cultures. Current protocols for isolating nasal epithelial cells imply long term incubation of epithelial cells which not only alters the phenotypical characteristics of hNECs, but may also change immune responses of hNECs<sup>(10-12)</sup>. Here, a short isolation protocol is described, i.e. a fast method for obtaining pure nasal epithelial cells with minimal effects on epithelial cells' phenotype and immunologic behavior.

One of the aims of this paper was to investigate the contribution of nasal epithelial cells to the production of IL-8, as IL-8 is known to be strongly up-regulated in CRSwithNP in parallel with typical Th2 cytokines like IL-5<sup>(22,23)</sup>. Our data do not point towards a major contribution of nasal epithelial cells to IL-8 production in NP disease. Both inferior turbinate hNECs of control and NP patients as well as hNECs from polyp tissue of NP patients produced similar amounts of IL-8 under baseline conditions. This observation is consistent with a study by Mullol et al.<sup>(33)</sup>, showing

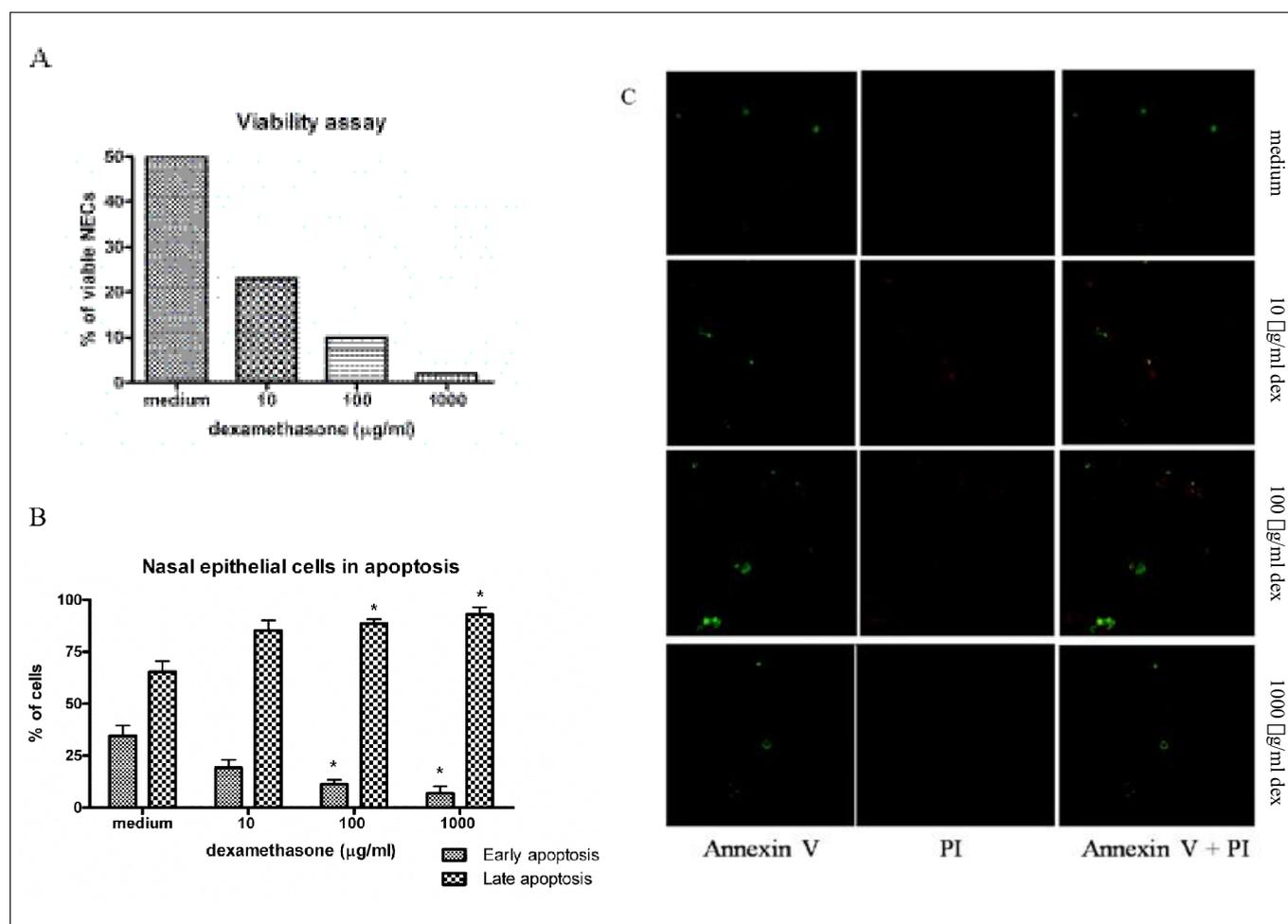


Figure 4. Apoptosis of hNECs. A) hNECs isolated from inferior turbinate of one healthy donor were incubated with increasing doses of dexamethasone for 24h and stained with trypan-blue. Number of viable cells decreases with increasing dexamethasone concentrations (representative graph from one patient). B) Graph representation of the counted early and late apoptotic cells. Four different fields were counted and apoptotic cells were differentiated into early (green) and late (green and red) apoptotic cells. Number of apoptotic cells induced by dexamethasone was compared to the medium (\*  $p < 0.05$ ). C) NECs incubated with increasing doses of dexamethasone for 6h and stained with annexin V-FITC (green) and PI (red). Annexin V-FITC+PI- cells are green and indicate early apoptosis whereas annexin V-FITC $\pm$ PI+ are green and red and indicate late apoptotic and already dead cells. Photographs were taken by confocal microscope.

no difference in production of IL-8, IL-6, GM-CSF and other cytokines between hNECs isolated from nasal mucosa of healthy donors and polyp tissue of NP patients. Therefore, IL-8 production by nasal epithelial cells does not discriminate between nasal polyp tissue and healthy mucosa.

It has been reported that pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  induce or modulate IL-8 expression in human airway epithelial cells<sup>(5,8,16)</sup>. Our data confirm that IL-1 $\beta$  strongly induces IL-8 production in human nasal epithelial cells from all three groups, whereas TNF- $\alpha$  has very little effect on hNECs in terms of IL-8 production. Similarly to the basal levels, IL-8 production induced by the pro-inflammatory cytokine IL-1 $\beta$  was not significantly different between hNECs isolated from nasal turbinate or polyp tissue either from healthy or NP patients. These results suggest that there is no intrinsic difference in IL-8 production between the epithelial cells from healthy mucosa or nasal polyp tissue, neither in unstimulated conditions nor upon stimula-

tion with pro-inflammatory cytokines. On the other hand, the local environment in NP tissue is highly inflammatory creating strong stimulus for hNECs to produce IL-8. This observation, together with the IL-8 production by eosinophils, which are highly present in NP tissue, may account for the higher levels of IL-8 in NP tissue compared to healthy nasal mucosa<sup>(23)</sup>. In our group, allergic status or usage of intranasal corticosteroids had almost no effect on the IL-8 production in hNECs.

Previously, it has been reported that hNECs from NP tissue produce more IL-8 than hNECs from healthy donors, which was reduced by dexamethasone treatment<sup>(7)</sup>. However, the same authors also showed no difference in IL-8 production between hNECs from healthy mucosa and NP tissue<sup>(33)</sup>. As mentioned earlier, no difference was observed here between hNECs from three sources in terms of basal IL-8 production. Interestingly, high dose of dexamethasone abolished the production of IL-8 whereas lower doses had almost no effect on IL-8 levels. We hypothesized that

the inhibitory effect of dexamethasone on IL-8 production at the lower doses is masked by the induction of apoptosis which might lead to the release and secretion of pre-synthesized IL-8 from an early apoptotic nasal epithelial cell. Higher doses of dexamethasone probably have a much stronger inhibitory effect, which cannot be influenced by the level of hNECs apoptosis. Indeed, it has already been shown that dexamethasone can induce apoptosis of airway epithelial cells in both humans<sup>(6,34)</sup> and mice<sup>(35)</sup>. Our results thus confirm that dexamethasone induces apoptosis of hNECs. hNECs incubated with dexamethasone lose their viability in a concentration-dependent manner and enter apoptosis faster than cells grown in the medium without dexamethasone. The mechanisms by which dexamethasone induces apoptosis of hNECs and release of IL-8 still remain to be explained. The effects of dexamethasone in terms of IL-8 secretion should probably be explored on the post-translational level or on the level of exocytosis of vesicles containing pre-synthesized IL-8. Proteins involved in degradation of IL-8 mRNA might also be affected by dexamethasone since it is known that dexamethasone strongly affects the stability of IL-8 mRNA<sup>(30)</sup>.

In conclusion, we here present a novel technique for epithelial cell isolation and culture, showing that IL-8 production does not discriminate between human nasal epithelial cells isolated from nasal turbinate or nasal polyp tissue of control and NP patients. In addition, dexamethasone induces apoptosis of hNECs with concomitant reduction of IL-8 secretion by hNECs.

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#### AUTHORSHIP CONTRIBUTION

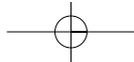
Major part of the research has been conducted in the host lab of C. van Drunen and W. Fokkens in the Amsterdam Medical Center. C. van Drunen, W. Fokkens and P. Hellings designed the research. P. Hellings and M. Jorissen harvested nasal tissue from healthy and NP patients. Isolation and stimulation of nasal epithelial cells was done by P. Hellings. I. Callebaut and V. Hox actively contributed in the experimental part of the study. First author performed experiments related to apoptosis assay, analyzed data and wrote the paper.

#### CONFLICT OF INTEREST

Authors declare no conflict of interest.

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