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Anti-inflammatory Effects of Polihexanide and Polyethylene Glycol in an *In Vitro* Study in Chronic Rhinosinusitis

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Abstract. *The majority of patients affected by chronic rhinosinusitis (CRS) suffer from eosinophilic infiltration. We hypothesised that polihexanide and polyethylene glycol as an antiseptic might alter the eosinophil-associated IL-5 and eotaxin-3 expression in CRS and also the expression of MMP-9, being involved in the tissue-remodelling in CRS. After obtaining samples from 10 CRS patients with (CRSwNP) and without nasal-polyposis (CRSsNP) and 2 patients with inverted-papilloma undergoing functional endoscopic sinus surgery (FESS), the expression of interleukin-5, eotaxin-3 and MMP-9 were evaluated by an ELISA assay with and without the tested agent. CRSwNP showed a significantly increased expression of IL-5. Polihexanide seems not to alter the attraction of eosinophils in patients with CRS via IL-5 expression. Also elevated levels of MMP-9 could not be reduced to normal values. But there exists statistically significant evidence that the self-amplification process of eosinophils via eotaxin-3 could be influenced by the administration of polihexanide.*

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal and paranasal sinus mucosa which estimates prevalence ranges widely, from 2 to 16% (16). Symptoms like congestion of the nose, nasal discharge, facial pain and reduced olfaction persist for more than 12 weeks and have a serious impact on the quality of life of these patients. Published data suggest that the quality of life is more severely impaired in patients suffering from CRS compared to patients with congestive heart failure (15) highlighting the importance of this ailment. The treatment-of-choice is the removal of the affected mucosa by functional endoscopic sinus surgery (FESS) (11). The alterations of the chronic inflamed mucosa range from inflammatory thickening to nasal polyps (2). The pathophysiology of CRS is still unknown, but the majority of patients suffer from eosinophilic infiltration with a subsequent dominant T-helper type-2 (Th2) cytokine profile (42). It is discussed that CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) are caused by totally different pathophysiological mechanisms (2). Another patho-physiological feature is the fact that CRSwNP is frequently observed in patients with asthma. Furthermore the two diseases also have a number of common pathological features: the activation of T-helper type-2 (Th2)-like lymphocytes and eosinophils secreting interleukin (IL)-3, IL-5, IL-13 and eotaxin (34-36, 47). IL-13 is elevated in sinus lavage of patients with CRS compared with controls (27). In consequence, IL-13 also affects the airway remodelling of the inflamed paranasal mucosa. IL-13 alters the function of matrix metalloproteases, which remodel the extracellular matrix (44). In former studies the application of IL-13 could induce a significant increase in β-catenin expression in eosinophilic CRS cell culture (36). β-catenin has been interpreted as a marker for the airway remodelling in CRS. Eotaxin is considered to be another crucial player in the regulation of eosinophilic inflammation and subsequent extracellular matrix remodelling in CRS. IL-5 is acknowledged to be responsible for the accumulation of eosinophils and furthermore the activation in nasal polyps (1). This finding is supported by the fact that IL-5 expression was observed in nasal polyps and inferior turbinates, but IL-5 was not detectable in the serum of patients suffering from CRS with nasal polyps (8). Another important aspect in the pathophysiology of CRS appears to be the detection of biofilms on the mucosa of CRS patients (12). These biofilms seem to colonise staphylococcus aureus (*S. aureus*). The chronic infection in the form of biofilms may play an important role in the maintenance of the recalcitrant inflammation in CRS. Therefore, understanding the relevance of the interaction between the biofilm, colonisation with *S. aureus* and the respiratory mucosa is an important task in the decipherment of the pathophysiology of CRS.

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Polihexanide is a local antiseptic, which is used in a broad variety of wound care products and perioperative cleansing products. It is effective against a wide spectrum of micro-organisms, exhibits lower cytotoxicity and lower levels of irritation than other antiseptics (18, 31). The duration of wound healing could be reduced by a dilution of 0.04% polihexanide in polyethylene glycol in an animal wound model (22).

We hypothesised that Polihexanide and polyethylene glycol administration might alter the IL-5 and eotaxin-3 expression in CRS and therefore reduce the process of inflammation. Furthermore this study was set up to determine the expression of MMP-9, being involved in the tissue remodelling in CRS and postoperative wound healing after FESS.

Materials and Methods

Patients, tissue collection and cultures. The cultures were obtained from ten patients suffering from CRSwNP (5 patients) and CRSsNP (5 patients), undergoing FESS at the Department of Otorhinolaryngology at the University Hospital Mannheim, Germany in 2006-2010. The cultures of the third group were gained from two patients undergoing FESS suffering from inverted papillomas.

A written consent was obtained from all patients for the use of tissue samples. The study was approved by the Ethics Committee of the Faculty of Medicine, Mannheim, University of Heidelberg, Germany according to the ethical rules for human experimentation that are stated in the 1975 Declaration of Helsinki. After the obtaining the sample, a sterile fibroblast culture of sinus mucosa was set up with each sample. A modified in-vitro culture according to Sauter *et al*. was used (36, 40).

Test agent. The test agent was Lavasorb® (Fresenius Kabi AG, Linz, Austria), which contains 0.4g Polihexanide (Polyhexamethylene Biguanide (PHMB)) and 0.02g polyethylene glycol 4000 (Macrogol®) in 1l Ringer solution. Macrogol reduces the surface tension of the solution and thus facilitates the humidification of the wound surface.

Incubation with Lavasorb®. All samples, except for an "untreated" control group, were treated with Lavasorb® in a further dilution of either 1:20 (1 ml in 20 ml Ringer solution) or 1:100 (1 ml in 100 ml Ringer solution).

After 24, 48, 72 and 98 h of incubation the expression of IL-5, eotaxin-3 and MMP-9 in the supernatants of the cultures were determined.

Cytokine immunoassay. The supernatants of the cultures were stored in sterile test tubes and saved at –20˚C until used. The expression of the examined interleukin, cytokine and proteinase were evaluated by an ELISA assay (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against the objectives. The exact products used were the following: DuoSet Human Total MMP9 Elisa/Order No. DY 911/R+D Systems/Minneapolis/USA. BD OptEIA Human IL-5 Elisa Set/Order No. 555202/BD Biosciences, Franklin Lakes, New Jersey, USA. BD OptEIA Human Eotaxin Elisa Set/Order No 555175/BD Biosciences, Franklin Lakes, New Jersey, USA.

According to the manufacturer, each ELISA assay determined IL5, eotaxin-3 or MMP-9 in 100 µl of supernatant. The cells were further grown in 96-well plates (Part 890218) with 12 strips of 8 walls coated with an antibody against either the proteins of interest. After 24-96 hours incubation, the expression of the markers of interest in the supernatants was determined.

Statistical analysis. Statistical analysis was performed with SAS (SAS/STAT; Version 8, SAS Institute Inc., Cary, NC, USA). An analysis of variance (ANOVA) - utilising the student's t-test and the Dunnet's procedure against the untreated control group (10) - was used to calculate *p*-values. As no Bonferroni adjustments were necessary, the level of statistical significance were set to $p<0.05$. Further statistical analysis and plotting was done using "R", an open source environment for statistical computing and graphics (32).

Results

Analysing the effect of incubation-duration (24, 48, 72 and 96 h), no statistically significant difference was found in the expression of interleukin-5, eotaxin-3 or MMP-9 in none of the tested conditions.

IL-5. Lavasorb® did not have a significant effect on the expression of interleukin-5 in any of the used concentrations (none, 1:20 or 1:100). In the group effects a significantly increased expression of IL-5 in CRSwNP (0.094 pg/ml ± 0.168 pg/ml) compared to the inverted papilloma group (0.044 pg/ml ± 0.045 pg/ml) can be seen ($p=0.048$)

The treatment with Lavasorb® did not alter the concentration of interleukin-5 in the cell cultures supernatant. Figure 1 shows the unchanged expression profile. The 3 different groups are marked with different colours. The 3 different groups were marked in different shades of grey.

Eotaxin-3. The used concentration (none, 1:20 or 1:100) of Lavasorb® had a significant effect on the expression of Eotaxin-3. The higher the concentration the more decrease in eotaxin-3 expression in all 3 groups could be shown. Figure 2 gives an graphical interpretation of the data and Table I summarises the expression changes. The *p*-values were far below $p=0.01$ in all of the groups and all of the treatments when compared to the untreated group, except Lavasorb 1/100 in the CRSwNP group. No significance could be detected.

MMP-9. MMP-9 was not significantly altered by the treatment with Lavasorb® in different concentrations (none, 1:20 or 1:100). Though CRS cell cultures showed a significantly increased expression level of MMP-9 compared to the control group (3.27 pg/ml ± 2.09 pg/ml) with $p<0.01$. The mean value of MMP-9 concentration in CRSsNP therefore was 34.23 pg/ml ± 39.06 pg/ml and the mean value of MMP-9 concentration in CRSwNP was 27.33 pg/ml ± 31.58 pg/ml. The box-whisker plots in Figure 3 depict the considerable increase in MMP-9 expression in the CRS groups.

Table I. *Eotaxin expression in cell culture supernatants.*

Group	Treatment	Eotaxin-3 exp.	Std. dev.
Control	untreated	3.25 pg/ml	±1.17 pg/ml
	Lava1/100	2.60 pg/ml	±1.08 pg/ml
	Lava1/20	1.27 pg/ml	±0.83 pg/ml
CRSsNP	untreated	3.94 pg/ml	±2.25 pg/ml
	Lava1/100	2.94 pg/ml	±2.05 pg/ml
	Lava1/20	1.82 pg/ml	±0.90 pg/ml
CRSwNP	untreated	3.97 pg/ml	±3.12 pg/ml
	Lava1/100	4.50 pg/ml	±3.84 pg/ml
	Lava1/20	2.20 pg/ml	±0.92 pg/ml

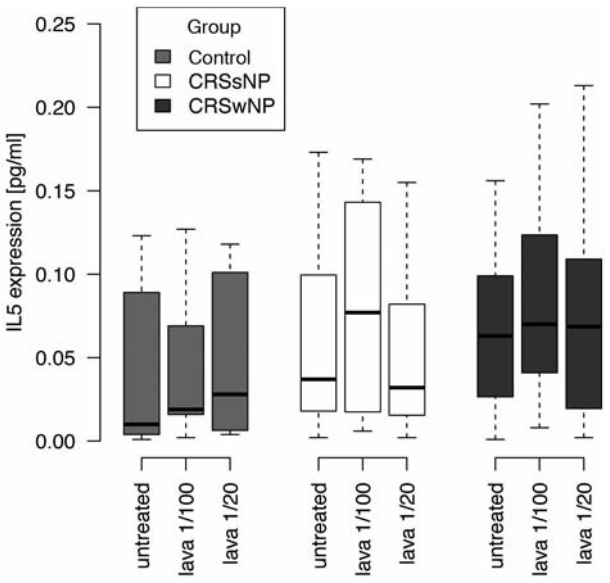

Figure 1. *Box-Whisker Plot of the IL-5 expression in correlation with the concentration of Lavasorb®. The boxes represent the interquartile range (IQR) with the whiskers extending up to 1.5 times the IQR. Outliers are marked as circles and the median is marked with a solid line.*

Discussion

All long-term wounds occur to have a certain level of bacterial burden. The imbalance of degradation by apoptosis and the reconstructive processes in long-term wounds facilitates the bacterial colonisation. *S. aureus* is one of the most problem-causing in surgical wound infections (39). *S. aureus* has direct cytotoxic effects to a variety of tissues, *e.g. S. aureus* was able to liberate pro-inflammatory cytokines in keratinocyte cultures. The keratinocytes continued to express the pro-inflammatory cytokines after being incubated with heat-inactivated staphylococcus species (46). This effect could be similar in respiratory mucosa. After FESS there is a prolonged mucosal wound healing. Furthermore colonisation of biofilms in the upper airway tract by *S. aureus* has been proven (20).

Polihexanide demonstrated its abilities to improve wound healing in infected and even in uninfected wounds, which indicates that its positive effects on wound healing are additional to its antiseptic effect (22).

IL-5. Interleukin-5 expression seems to be unchanged by the applied concentrations of Lavasorb®. Nevertheless the CRSwNP group had a significantly increased expression of IL-5.

IL-5 (also called eosinophil differentiation factor), is a cytokine being involved in the control of eosinophilpoiesis. Furthermore eosinophil growth and activation is regulated with its expression (4). Many eosinophil-mediated diseases like asthma, rhinitis, and eosinophilic esophagitis are therefore linked with increased levels of IL-5 expression (28). IL-5 also seems to play a major role in CRS. Significantly elevated levels in CRSwNP-patients have been reported before (7). This matches the data presented here. These high levels of IL-5 in nasal polyps have been detected at the mRNA and protein levels (33).

We could not detect a significant alteration of IL-5 expression after Lavasorb incubation. In the literature a local antiseptic therapy caused a reduction of bacterial colonisation (9). Thus consequences on the IL-5 expression have been anticipated.

Furthermore a reduction of IL-5 in nasal secretion was described after methylprednisolone therapy in CRS patients (43). Yet, a systemic therapy with antibiotics failed to show a reduction in IL-5 expression (43). Nevertheless the reduction of IL-5 appears to be consistent in CRSwNP due to the fact that a study with anti-IL-5 demonstrated that a single injection reduced nasal polyp scores only in half of the treated patients for 4 weeks. But the responders had increased IL-5 concentrations in nasal secretions at baseline compared with non-responders, and logistic regression analysis revealed that increased nasal IL-5 levels (>40 pg/ml) predict a possible response to anti-IL-5 treatment (13). Another explanation for our results could be that the surface of the cell cultures is – compared to the sinus mucosa – not broad enough to reveal alterations in eosinophilpoiesis.

Eotaxin-3. Eotaxin - also called chemokine (C-C motif) ligand 26 (CCL26), macrophage inflammatory protein 4-alpha (MIP-4-alpha) or thymic stroma chemokine-1 (TSC-1) – is an eosinophil-specific β-chemokine. In experiments by Shinkai *et al.* it was demonstrated that Eotaxin-3 is involved in the accumulation of leukocytes, especially eosinophils, at sites of inflammation. The hypothesis they published states that eosinophils are activated by eotaxin-3 expressed on vascular endothelial cells, extravasate from the bloodstream to an inflamed tissue, and then migrate to the centre of the tissue through the action of eotaxin (38). Due to this finding it is assumed that Eotaxin is an important key protein in the

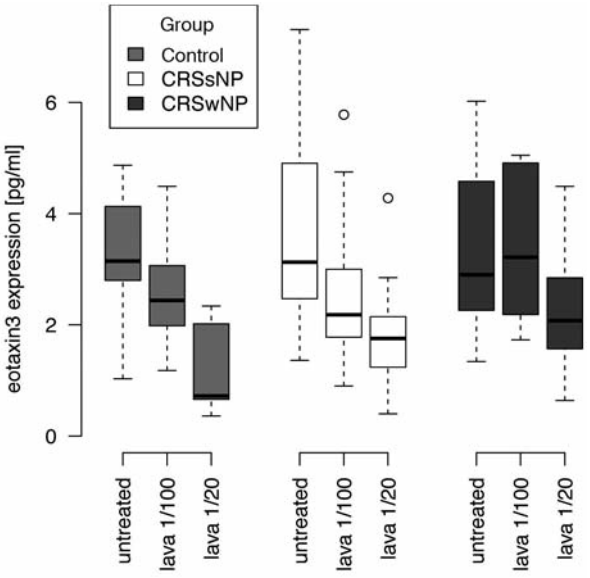

Figure 2. *Box-Whisker Plot of the eotaxin-3 expression in correlation with the concentration of Lavasorb®. The boxes represent the interquartile range (IQR) with the whiskers extending up to 1.5 times the IQR. Outliers are marked as circles and the median is marked with a solid line.*

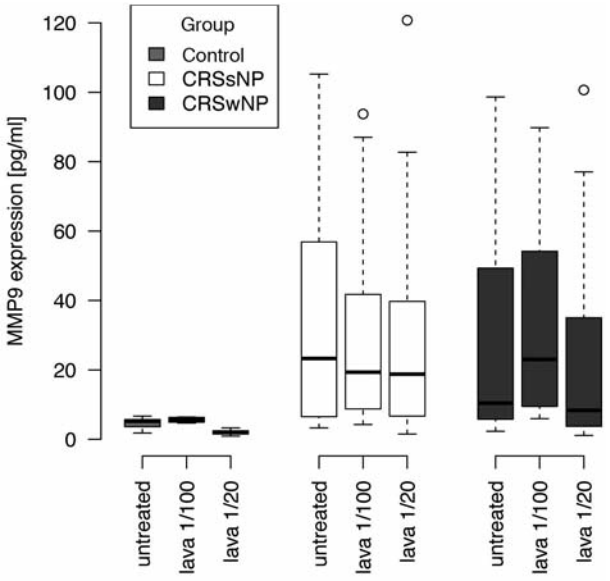

Figure 3. *Box-Whisker Plot of the MMP-9 expression in correlation with the concentration of Lavasorb®. The boxes represent the interquartile range (IQR) with the whiskers extending up to 1.5-times the IQR. Outliers are marked as circles and the median is marked with a solid line.*

pathophysiology of eosinophilic inflammatory diseases such as atopic dermatitis, allergic rhinitis, asthma and parasitic infections (17).

In contrast to IL-5, polihexanide had a significant effect on the expression of eotaxin-3. The decrease in eotaxin-3 expression was proportional to the concentration of Lavasorb®. This effect was detected in almost all groups (control, CRSsNP, CRSwNP) with the exception of Lavasorb 1/100 in the CRSwNP group, where no significant effect was observed. Interestingly we could not see a group effect neither in CRSsNP nor in CRSwNP, as expected regarding to other publications (17, 47). As Molinaro *et al.* suggested, eosinophilia in nasal polyps may be a self-amplification process whereby increasing numbers of eosinophils are recruited to enter the polyp as a result of production of eotaxin by eosinophils already within the polyp (29). The reason, why the drop of eotaxin-3 expression with increasing concentrations of polihexanide is not simply a destruction of the cell culture, is that IL-5 and MMP expression remains unchanged. So, one can hypothesise that polihexanide, which has a proven effect on transmigration on neutrophils (37), also has a modulatory effect on eosinophils, especially on their expression of eotaxin.

As stated above, eosinophilic esophagitis is a disease strongly connected to an eosinophil reaction. Caldwell *et al.* reported on increased eotaxin-3 levels in this disease and the possibility to alter them by topically administering glucocorticoides (3). Glucocorticoides are well-known in the treatment of CRSwNP but not all patients are responding to the treatment (25). Maybe polihexanide could be a new therapeutic option for those glucocorticoide-insensitive patients.

MMP-9. The concentration of MMP9 was tremendously elevated in CRS patients compared to the control group, regardless if they had nasal polyps or not. On the other hand the expression was not significantly altered through treatment with polihexanide.

Metalloproteinases play a crucial role in tissue remodelling and are of particular interest in the research of airway diseases. Tissue remodelling of the effected mucosa includes the break down and repair of tissue structures and interstitial stroma. The MMPs are proteolytic enzymes responsible for remodelling of mainly the extracellular matrix (ECM). There are many different subgroups, which share important common properties; one is that they require an active Zn^{2+} site for their catalytic function (45). One of the main sites, where MMPs are active, is the respiratory mucosa. MMP-9 is mainly active in degrading gelatine, elastin, aggrecan, and collagens. Nevertheless its exact role in polyp and pseudocyst formation remains unclear (26). Diseases with elevated concentration of MMP-9 in the ECM are lung cancer, asthma (6), allergic reactions (21, 41), or lung trauma (5, 14). Also endothelial and inflammatory cells in nasal polyps have shown elevated concentrations of MMP-9 (23, 24). Our results are matching these studies. The missing significant difference between CRSwNP's and CRSsNP's patients in

MMP-9 expression is also in line with another published study (45). Lavasorb® with its antibacterial and possible immunomodulatory effects did not have an impact on MMP-9 expression in any of the used concentrations.

Moor *et al*. who published a study on polymicrobial colonised chronic wounds found a seven-fold excess of MMP-9 in wound fluid compared to tissue comparable to the CRS cultures (30). This may suggest that Lavasorb® with it's antimicrobial potential is an ideal candidate to treat CRS-patients and the biofilm in their sinuses. However polihexanide seems not to have the ability to stop the remodelling process in CRS. So, the role of biofilms and the effect of their break-up remains unclear, as various studies only report a "certain" impact on CRS (19, 48). In contrast to the eosinophil reaction provoked by eotaxin-3, the remodelling of the extracellular matrix seems not to be altered by the presence of Polihexanide.

In conclusion polihexanide appears not to alter the attraction of eosinophils in patients with CRSwNP or patients with CRSsNP *via* an IL-5 expression. Additionally, elevated levels of MMP-9 and therefore the process of tissue remodelling could not be reduced to normal values using this local antiseptic. On the contrary there is statistically significant evidence that the self-amplification process of eosinophils in the nasal mucosa could be influenced by the administration of Lavasorb®. Higher concentrations (1:20) seem to produce better results without inhibition of cell culture growth. Eotaxin-3 is down-regulated significantly in all of the 3 patient groups. This way intractable eosinophil infections of the nasal mucosa may be stopped or at least a relief of symptoms may be achieved. As Lavasorb is already approved for use on human mucosa, the next logical step should be to perform clinical studies with the topical administration of Lavasorb® in patients with CRS.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

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